SEQUENTIAL INJECTION ANALYSIS (SIA) TECHNIQUE FOR THE ASSAY OF KETOCONAZOLE AND DICLOFENAC SODIUM IN DRUG FORMULATIONS WITH CHEMOMETRIC APPROACH

BY

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This thesis, written by Hatim Dafalla Mohamed Dafalla under the direction of his thesis advisor and approved by his thesis committee, has been presented to and accepted by Dean of Graduate Studies, in partial fulfillment of the requirements for the degree of

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THESIS ABSTRACT

NAME: **HATIM D. MOHANED**

TITLE:

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MAJOR FIELD: **CHEMISTRY**

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This research work describes newly adopted Sequential Injection Analysis (SIA) methods with a spectrophotometric detection for the quantitative determination of Ketoconazole and Diclofenac sodium in drug formulations. Different chemometrics optimization methods, namely: uni-variate, factorial design, and multisimplex have been utilized. The results obtained from the adopted SIA methods were found to be in agreement with the results obtained from the British and US pharmacopoeia Standard method thus documented the accuracy of the newly adopted methods. The methods are initially validated following the USP30-NF25 procedure. The SIA has the additional advantage over the previously reputed methods in reducing cost by minimizing time, amount of reagent consumed, man power required and improved accuracy of analysis due to computer aided analysis procedure

ملخص الرسالة

الاســـــــــــم: حاتم دفع الله محمد دفع الله

عنوان الرسالة: تقنية التحليل بالحقن المتتابع لتعيين كمية الكيتوكونازول والديكلوفيناك صوديوم فى التركيبات الدوائية باستخدام الكيموميتركس.

التخصـــــــص: كيمياء تحليلية

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هذه الدراسة تصف تقنية التحليل بالحقن المتتابع باستخدام الكشف الطيفي كطريقة جديدة لمعرفة كمية المادة الفعالة لكل من الكيتوكونازول المضاد للفطريات، والديكلوفيناك صوديوم المضاد لالتهاب المفاصل أو الجروح الحادّة باستخدام طرق كيموميتركية مختلفة تحديدا: طريقة المتغير الواحد ، طريقة تصميم التجارب ، وطريقة التحقق الامثلية. النتائج التى ظهرت عن طريق استخدام وسيلة (SIA) كانت مطابقة للنتائج التى ظهرت عن طريق استخدام وسيلة دستور الأدوية الأمريكي (USP) والبريطانى (BP). النتائج التى حصل عليها باستخدام الطريقة الجديدة تم التحقق منها باتباع طريقة (USP30-NF25). بالاضافة الى ذلك فان استخدام طريقة (SIA) قد اظهرت ايجابيات اضافية مثل اختصار الوقت ، والمواد الكيميائية ، والطاقة البشرية للقيام بالعمل.

CHAPTER 1

INTRODUCTION

1.1 PHARMACEUTICAL ASSAY METHODS AND AUTOMATION

The SI analysis technique is now well known to have several advantages over conventional techniques followed in the past in the analytical laboratories. The proponents of this work have recently introduced FI analyzer and FI/SI analyzer systems in the research laboratory at the Department of Chemistry, College of Science, KFUPM the technique could be taught theoretically and practically as a part of a core course entitled "Instrumental Chemical Analysis" offered to our undergraduate students. Therefore, the newly provided methods could be applied as standard methods in the practical syllabi of instrumental chemical analysis in our Department. The implementation of SI analysis to modern analytical laboratories is an essential addition and a significant development to suit the new millennium expected changes and enhancements. Pharmaceutical industry is now growing in the Kingdom of Saudi Arabia with a special attention from the government and SI analysis would be a positive addition to meet the requirements. Environmental concerns and personnel safety as well as need for keeping operational cost to a minimum necessitate minimizing or eliminating such waste. Waste generation cannot be eliminated in the near future since traditional assay methods cannot economically be

replaced. However, the SI technique is well suited to this goal, which is highly targeted by the government of the Kingdom.

Traditionally, pharmaceutical analysis relies heavily on chromatography, yet the assay of pharmaceuticals also frequently requires the use of reagent-based techniques with spectrophotometric, fluorescence or electrochemical detection. Automation and miniaturization of these solution-based assays to make them fast and efficient are essential for many routine and research tasks in pharmaceutical laboratories. Ideally, instrumental techniques should be versatile, capable of accommodating a wide variety of assays without the need for system reconfiguration, and compatible with both optical and electrochemical detectors [1]

Automation is a key demand of modern industrial-scale pharmaceutical analysis. New product development, validation of manufacturing process and routine quality control involve the analysis of a considerable amount of samples in order to ensure compliance of the formulations to the limits established by international authorities. Typical quality control tests that involve the analysis of many samples include [2]:

(1) Assay of final products.

(2) Dissolution profiles during production and validation of manufacturing processes.

(3) Dosage uniformity tests.

(4) In-process control especially during blending uniformity for solid and semi-solid formulations.

1.2 OBJECTIVES

1. To adopt novel methods for the assay of two compounds

A. Ketoconazole (anti-fungal) by oxidizing it with cerium (IV) in sulfuric acid media and spectrophotometerically monitoring the oxidized form of the drug.

B. Diclofenac sodium (anti-inflammatory) by oxidizing it with permanganate in sulfuric acid media and spectrophotometerically monitoring the appearance of the brown colored product resulting for quantitative assay of the drug.

2. SIA technique will be acquainted with and applied as a tool for the assay methods.

3. Validation of the newly adopted methods will be carried out following USP monograph.

4. Advantages of the newly adopted methods will be highlighted.

1.3 FLOW INJECTION ANALYSIS

Flow injection analysis (FIA) is a well-established automated technique with numerous and widespread applications in quantitative chemical analysis. In brief, a typical FI setup involves injection of a define volume of the sample into a moving stream of a solution which serves as a carrier and propellers the sample zone to a flow-through detector. Between the injection and detection points, the analyte of interest is chemically or physically transformed to detectable specie. FI is generally a simple and inexpensive technique employing common instrumentation such as peristaltic pumps and low-pressure injection valves. Compared to batch methods it offers increased sampling rate, lower reagents consumption, better precision and high versatility. The above-mentioned advantages of FI have led to a continuously Increasing interest in pharmaceutical analysis and quality control applications [3] The main chemical approach in applying FI to pharmaceutical analysis is through automation of derivatization reactions. On the other hand, more complicated procedures such as on-line solid-phase and solvent extraction, optical sensors, enzymatic reactions, etc., tend to attract scientists, based on the growing need for more sensitive and selective analytical methodologies. Although FI can be coupled to any detection system capable of flow-through operation covering a range that extends from simple LED-based optical systems to sophisticated mass spectrometers, UV–vis spectrophotometry seems to be the technique of choice for FI pharmaceutical applications. UV–vis spectrophotometry offers the advantages of simple, low cost instruments that are available at all laboratories. Running costs are minimal and no

highly-skilled personnel are required for their operation. Another significant advantage is that pre-existing batch instruments can be easily converted to flow-through by either home-made or commercially available cells. Apart from the monograph published by Calatayud in 1996 [3], recent reviews on the application of FI to pharmaceutical analysis cover the topics of multicomponent determinations [4] and amperometric detection [5].

1.3.1 Sequential Injection Analysis

SIA (Sequential Injection) analysis is the second generation of FI (Flow Injection) techniques. It is a fully-automated approach to solution handling that allowing automating manual wet chemistry procedure in a rapid, precise, and efficient manner. Small solution zones are manipulated under controlled dispersion conditions in narrow bore tubing. While, like the first generation of FI technique, FI analysis, it is fundamentally dependent on the dispersion of zones in a flowing stream; conceptually, the practice of SI analysis is different from FI analysis. The upper diagram in figure 1 shows the main components of FI analyzer manifold and the lower diagram is for SI analyzer manifold.

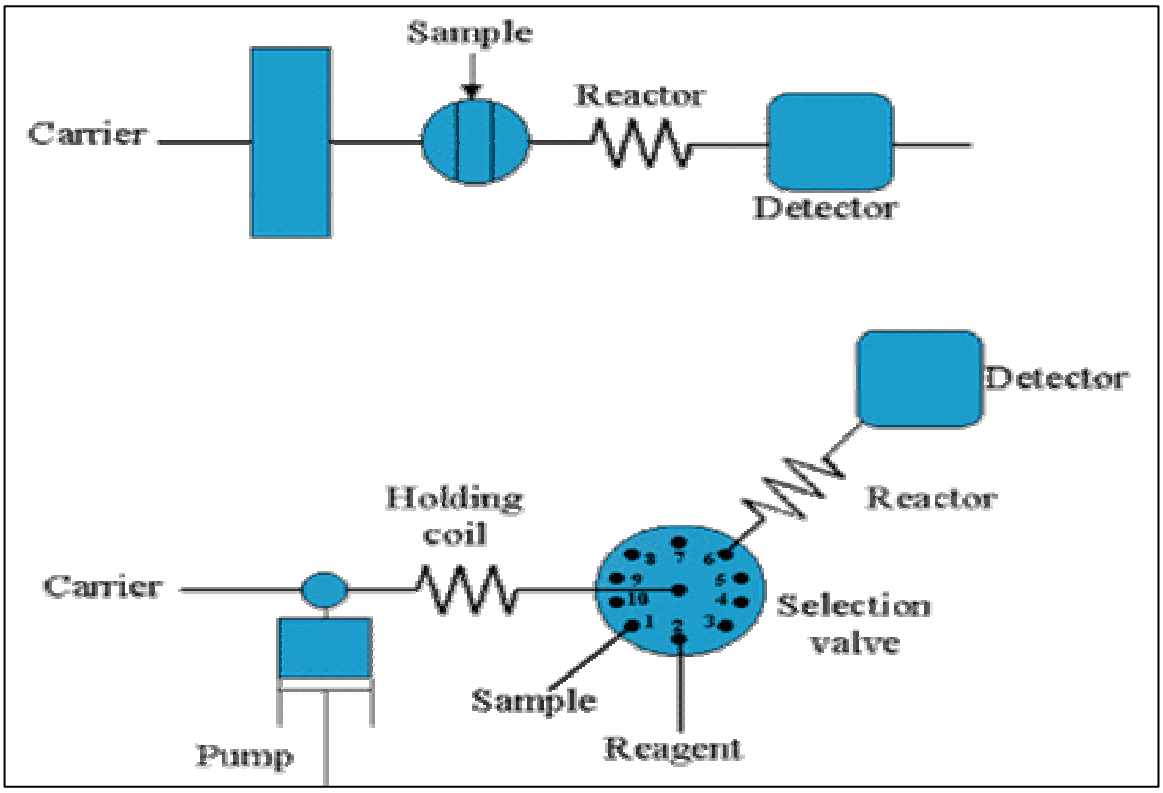


Figure 1: The major components of FI and SI analyzers

Consider a single line FI analysis experiment, where sample is injected into a carrier stream containing a reagent and sample is pumped into the sample loop of a two-position injection valve and the carrier is flowing constantly through the detector. The length of the sample loop determines the volume of sample injected. When the sample loop is loaded, the valve is switched on and the sample is introduced into a flowing carrier stream. The carrier carries the sample through the reactor (usually a reaction coil) to the detector. En route, the sample reacts with the reagent to form a detectable species that gives rise to a peak when it passes through the flow cell of the detector. A calibration curve is then used with the peak height, area or width to determine the concentration of the analyte in the solution. SI analysis, on the other hand, does not make use of an injection valve. Rather, a multi-positions selection valve replaces the injection valve. Usually, the peristaltic pump used in FI analyzer is replaced with a syringe pump in SI analyzer and an additional coil called the holding coil is added. To achieve the same measurement as described above, the syringe is filled with carrier solution containing the reagent. Then the selection valve is advanced to a port that is connected to the sample line. A small volume of sample is drawn up into the holding coil. The flow program determines the volume of sample; viz. the volume of sample that is drawn up by the pump into the holding coil. The selection valve is then advanced to a port that is connected to the detector, and the carrier transports the sample through the reactor to the flow cell of the detector. Again, a detectable species is formed and is registered as a peak by the detector. The concentration of the analyte in the solution is determined in a similar manner as for FI analysis.

It is worth noting that at the moment of injection in FI analysis experiment, an un-dispersed plug of sample is introduced into the carrier stream. In SI analysis, already during aspiration of the sample into the holding coil, dispersion begins to take place and the flow reversal, which takes place when the sample is sent off to the detector, plays a dramatic role in mixing the sample with the carrier. Fig. 2 depicts graphically what happens at the point of sample injection in an FI analyzer manifold (top) and during the flow reversal inherent to SI analyzer manifold (bottom) and a few seconds after the carrier has started to move the sample towards the detector. This phenomenon may cause SI analysis peak to look slightly different to FI analysis peak. As long as there has been good mixing between sample and reagent, this will not affect quantification because samples and standards are treated alike. In fact, it has been shown that the flow reversal contributes significantly to the mixing of zones.

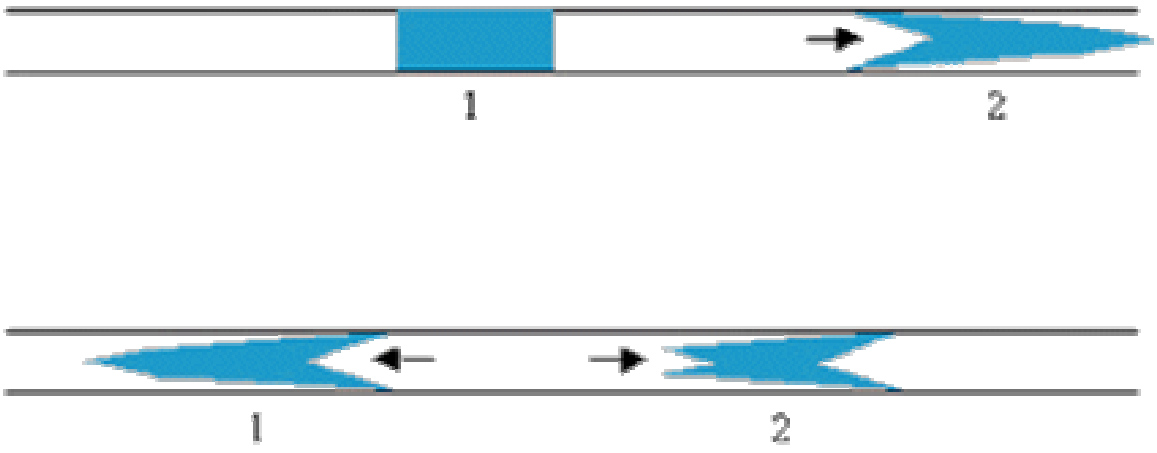


Figure 2: Movement of injected solution through the channels of FI analyzer manifold (top) and SI analyzer (bottom)

More often than not, instead of including the reagent in the carrier, an SI analysis experiment is expanded so that the reagent is loaded as a separate zone. In this case, the syringe is first filled with a simple carrier or buffer. After the sample zone has been drawn up into the holding coil, the selection valve is advanced to a port connected to a reagent reservoir and a small reagent zone is drawn up into the holding coil. Through this way, it is possible to construct a stack of well-defined zones, which can be mixed together to give a detectable species. You will appreciate that unlike FI analyzer, which requires re-plumbing when a more complex chemical addition scheme is required, in SI analyzer, all that is, required is a change to the flow program. The manifold remains the same. The additional advantages of lowering reagent consumption and minimizing the production of potentially hazardous wastes are these important advantages of SI analysis.

The other ports of the selection valve can be used for calibration standards, additional reagents and as locations where more sophisticated operations such as dilution, trace enrichment, and incubation of reactants can take place.

A variation of SI analysis is sequential injection titration (SIT). In SIT, the reactor and detector in figure 1 are replaced with a stirred titration cell. Appropriate sensors, electrochemical or colorimetric, are placed in the titration cell, which acts as flow cell as well as titration chamber.

SIA was developed by Ruzicka and Marshall at the University of Washington in response to an industry-initiated requirement for a more robust automated wet chemistry technique than FI analysis. At the Center for Process Analytical Science (CPAC), industry representatives challenged faculty and researchers to take FI analysis to the next level.

Develop it to the point where its strengths were maintained and enhanced, but its limitations in the process environment were remedied. At other institutions, the problem was tackled by investigating the use of sensors. Other researchers explored miniaturization of FI analyzer.

While SI analysis makes use of a simpler flow manifold (this is particularly so for multi component chemistries), development of the SI analysis method is not as straightforward. Careful attention needs to be given to the design of the measurement sequence to ensure that adequate zone penetration has taken place.

SI analysis has several advantages over FI analysis. Reagent use is drastically reduced. Typical FI analysis experiments make use of at least 3mL of reagent per measurement. SI analysis typically makes use of maximum 50 µl.

Flow manifolds are simple and robust typically comprising a pump, selection valve, and detector connected by tubing. The same manifold can be used for widely different chemistries simply by changing the flow program rather than the plumbing. Analyzer maintenance is therefore simplified. The selection valve replaces the injection valve and provides a means for selecting different sample streams and celebrants. This enables convenient automated calibration. Components used in a SI analyzer manifolds are amenable to laboratory, field, and plant operation. In addition to this, SI analysis enjoys all of the benefits of FI analysis. [6, 7, 8, and 9]

Table 1: Major differences in the instrumentation of FIA and SIA

SIA	FIA
It consist of a single channel, high precision bi-directional pump called the syringe pump ,a holding coil, multiselection valve, reaction coil and a detector	It consists of a high quality multichannel peristaltic pump, an injection valve, a coiled reactor and a detector.

1.3.2 The Advantages and Disadvantages of SIA

While SIA makes use of a simpler flow manifold (this is particularly so for multi component chemistries), development of the SIA method is not as straight forward. Careful attention needs to be given to the design of the measurement sequence to ensure that adequate zone penetration has taken place. Accurate measurement of sample and reagent zones necessitates microprocessor control. Of course, once the method has been developed, the microprocessor ensures slavish repetition of the optimized sequence. Previous limitations associated with the use of syringe pumps (notably the need for a syringe fill cycle, and poor precision for sample volumes smaller than 10 µl), no longer apply. Global FIA has developed a new pump called the milliGAT^{TM}. That has all the advantages of a syringe pump but eliminates many of its limitations. In particular, the fill cycle and need to compromise syringe diameter in order to allow sufficient carrier volume for an experiment are conveniently overcome in the milliGAT^{TM}

1.3.3 Advantages of SIA over FIA.

1. Reagent use is drastically reduced. Typical FIA experiments make use of at least 1mL of reagent per measurement. SIA typically makes use of 50 µl. This means that in a 24-hour period assuming one measurement per minute, the FIA analyzer would consume 1450 ml of reagent. The SIA analyzer would consume 72 ml. It has been noted that the most frequent reason for process analyzer failure is running out of reagents.

2. Flow manifolds are simple and robust typically comprising a pump, selection valve, and detector connected by tubing. The same manifold can be used for widely different chemistries simply by changing the flow program rather than the plumbing. Analyzer maintenance is therefore simplified.
3. The selection valve replaces the injection valve and provides a means for selecting different sample streams and calibrants. This enables convenient automated calibration.
4. Components used in a SIA manifolds are amenable to laboratory, field, and plant operation. In addition to these, SIA enjoys all of the benefits of FIA

1.4 CHEMOMETRICS

Since its introduction in 1974 [10, 11], flow injection analysis (FIA), has developed rapidly and in various directions. Today, it is an internationally approved, routine method for the determination of quite a large number of analytes [12–16], and is also used in sample pre-treatment for selective detectors [17–19], process control [20,21], drug dissolution testing [22] and hyphenated separation techniques [23,24], to mention just a few further applications. New applications and technical advances of the technique are being continuously reported, and the number of published FIA papers now exceeds 12,000 [25]. Its further development beyond the point of maturity and widespread acceptance has fostered versatile off-shoots, the most well known being sequential injection analysis [26] and lab-on-valve methodology [27]. These two advances show that FIA, not only provides a reproducible means of transporting diverse samples to any type

of detector, but it is also an ideal technique for mechanising tedious manual sample handling steps in the analytical chain. Chemometric techniques, chemometrics, were introduced into analytical chemistry almost in parallel with flow injection analysis [28, 29] and they have shown similar tendencies to FIA to increase in use and scope. Of most interest here is that chemometric approaches have been used in some cases during the development, refinement and validation of FIA methods, since they can save time during method development, and the treatment of data produced by some detectors in FIA systems more or less requires such approaches to yield analytically useful data. However, the majority of published FIA papers still report method development and detector signal treatments that do not employ any chemometrics at all. The reason for this is, perhaps, that most FIA practitioners have not yet discovered the inherent potential of the two scientific disciplines working hand in hand. This paper will therefore describe ways in which chemometrics can enhance the scope and power of FIA by considering a few representative cases where it is not immediately apparent that using chemometrics would improve performance. It is not intended to be a comprehensive review of the topic since several such reviews have already been presented [30, 31].

1.4.1 Definitions of chemometrics

There are many different definitions of chemometrics, but the one introduced by Massart et al. [32] reads: “Chemometrics is a chemical discipline that uses mathematics, statistics and formal logic

(a) To design or select optimal experimental procedures;

(b) To provide maximum relevant chemical information by analysing chemical data; and

(c) To obtain knowledge about chemical systems.

This definition is attractive since it specifically recognises and includes the use of experimental design for system optimisation. Applying this definition to a typical FIA system we can see that there are (at least) three stages in method development where chemometrics could be involved: method optimisation, signal evaluation and the subsequent calibration procedures.

1.4.2. Experimental design versus one-variable at-a-time approaches

"We optimized our FIA system. First, we established the optimum pH (6.5), and second, the lengths of the mixing coil (50 cm)". Unfortunately, statements of this kind are frequently found in FIA papers. This commonly used optimisation strategy is based on varying one variable at a time. The obvious weakness of this strategy will be shown by a simple example. Assume that we first investigate the influence of pH on the response (peak height). We select a coil length of 40 cm and vary the pH of the reagent and carrier solutions in the range 3–7 and obtain a maximum response at pH 6.5, see Fig. 3a. This pH value is then taken as an "optimum" value and used throughout the subsequent method development process. Next, the effect of varying the coil length is investigated in the range 30–70 cm. A maximum appears when using a coil length of 50 cm, consequently the combination pH 6.5 and 50 cm will be our choice for obtaining the final optimum conditions. Fig. 4a and b illustrate how we indeed reach a maximum at pH 6.5, and a maximum when using a 50 cm coil, respectively. However, the position of the true

maximum is given in Fig. 4c. This position, defining the ideal conditions as pH 7.8 and coil length 60 cm, would have been found if experiments had been based on techniques such as factorial design [33] or Simplex optimisation [34].

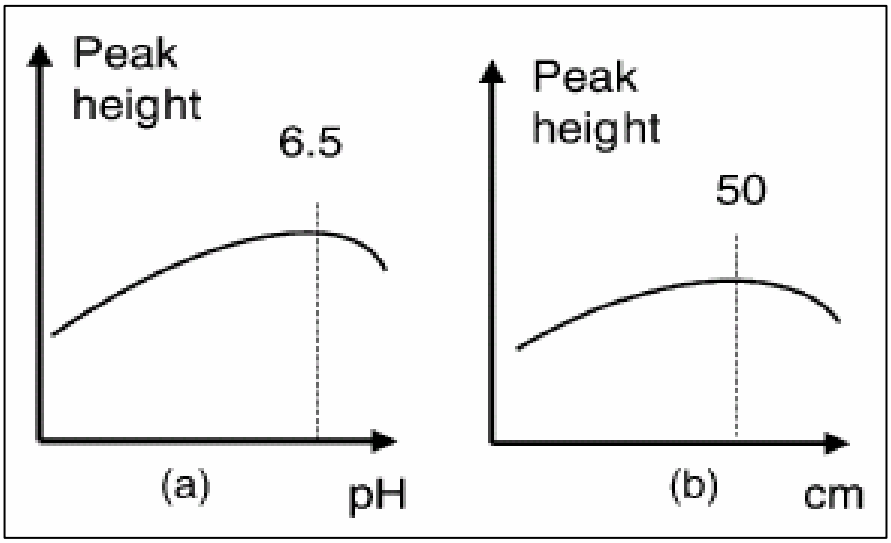


Figure 3 Optimization of an FIA system using the one-variable-at-a-time approach (a) peak height plotted versus pH giving an "optimum" at pH 6.5 and (b) peak height versus coil length giving an "optimum" at 50 cm.

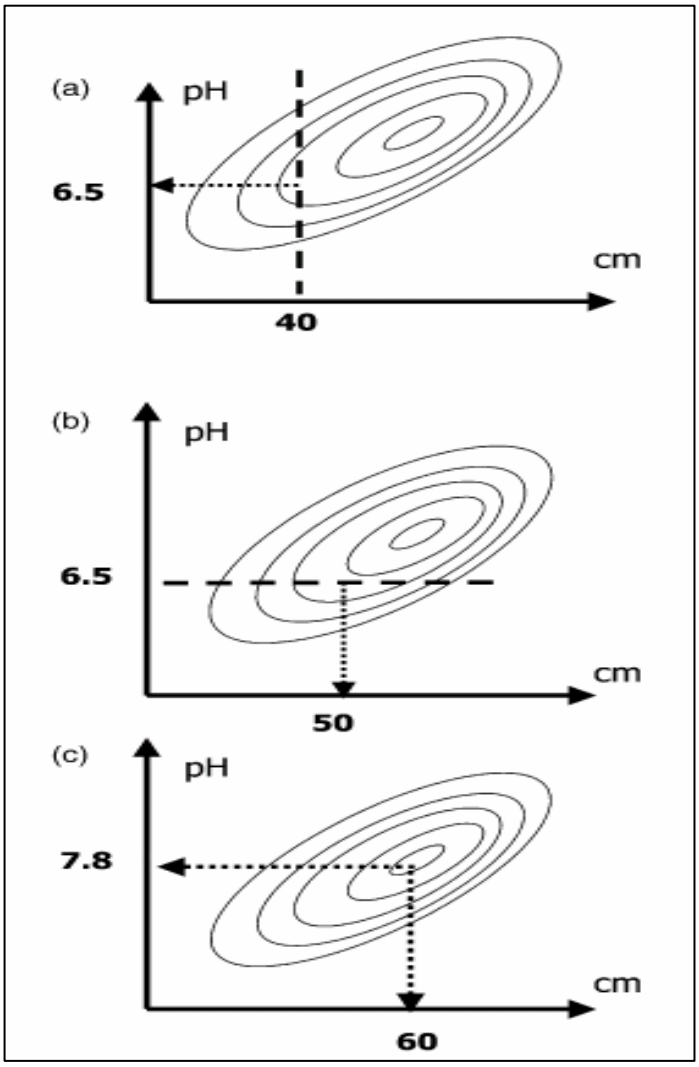


Figure 4 Optimization of an FIA system using the one-variable-at-a-time approach: (a) variation of pH using a coil length of 40 cm giving an "optimum" at pH 6.5; (b) variation of coil length at pH 6.5 giving an "optimum" at 50 cm; and (c) depiction of the "true" optimum at pH 7.8 uses a coil length of 60 cm

1.4.2.1 Defining the response functions

The fundamental question is: what is going to be optimised in the FIA system? The most commonly used response function is peak height, i.e. one often strives to maximise the detectability of the FIA system. Other relevant response functions are reagent consumption, response time and sample throughput. All these response functions are rather "coarse". Any one with at least some experience of FIA method development knows that maximum peak heights are obtained when the sample volume is large, the carrier flow is high and the reagent flows are low. All this assumes that the chemical reactions involved are fast enough to reach completion by the time of detection. Another obvious consideration is that if minimal reagent consumption is desired, then this optimum will appear when the pumps are off, so this response function is useless when applied in isolation. Practical limits are always set for the controlled variables, and other phenomena may appear that have to be taken into account, for instance, baseline drift, deteriorating reproducibility of the peaks, and changes in peak shape, to mention just a few possible complications. Surprisingly little has been done to develop practical and imaginative response functions in this context. For example, the "efficiency" of an FIA system might be expressed as peak height per microliter injected sample. For most FIA systems the peak height increases almost linearly with the injected volume up to about 100 µl, it then levels off and reaches a saturation value at about 400 µl. If this proposed response function is used, an optimum injection volume would probably fall in the range 100–200 µl. Larger volumes will undoubtedly produce higher peaks, but the "efficiency" defined in this manner will drop dramatically. The lack of reliable but creative response

functions might to some extent explain the low interest in using experimental design when developing new FIA methods. In this respect, some input might be obtained from papers describing the optimisation of chromatographic systems [35–38].

1.4.2.2 Variable selection

The number of variables that have to be optimized in a typical FIA system is often very large. For instance, the standard manifold used for the determination of ammonia according to ISO [13] comprises at least 36 variables [39]. Even if each of these 36 variables is varied at only two levels, high and low, there will be an incredible number of possible permutations of variables. If one experiment takes 1 min to perform and rebuilding the system to test a new permutation of variables also takes 1 min, it would then take 6,271,454 years to investigate all the possible permutations. Thus, it goes without saying that variables must be selected so that non-significant variables can be kept constant when optimising the remaining significant variables. Ideally, experimental design will be used in two steps. In the first step all variables will be included in a two-level Plackett–Burman design [40], to identify significant variables based on results obtained from a relatively limited number of experiments [41]. In the second step the significant variables will be investigated using, for instance, a full or reduced factorial design, keeping the non-significant variables at a constant level.

1.4.3 Experimental Design

Design of experiments was invented by Ronald A. Fisher in the 1920s and 1930s at Rothamsted Experimental Station, an agricultural research station 25 miles north of London. In Fisher's first book on design of experiments1 he showed how valid conclusions could be drawn efficiently from experiments with natural fluctuations such as temperature, soil conditions, and rain fall, that is, in the presence of nuisance variables. The known nuisance variables usually cause systematic biases in groups of results (e.g., batch-to-batch variation). The unknown nuisance variables usually cause random variability in the results and are called inherent variability or noise. Although the experimental design method was first used in an agricultural context, the method has been applied successfully in the military and in industry since the 1940s. Besse Day, working at the U.S. Naval Experimentation Laboratory, used experimental design to solve problems such as finding the cause of bad welds at a naval shipyard during World War II. George Box, employed by Imperial Chemical Industries before coming to the United States, is a leading developer of experimental design procedures for optimizing chemical processes. W. Edwards Deming taught statistical methods, including experimental design, to Japanese scientists and engineers in the early 1950s2 at a time when "Made in Japan" meant poor quality. Genichi Taguchi, the most well known of this group of Japanese scientists, is famous for his quality improvement methods. One of the companies where Taguchi first applied his methods was Toyota. Since the late 1970s, U.S. industry has become interested again in quality improvement initiatives, now known as "Total

Quality" and "Six Sigma" programs. Design of experiments is considered an advanced method in the Six Sigma programs, which were pioneered at Motorola and GE.

1.4.3.1 Fundamental Principles

The fundamental principles in design of experiments are solutions to the problems in experimentation posed by the two types of nuisance factors and serve to improve the efficiency of experiments. Those fundamental principles are

• Randomization

• Replication

• Blocking

• Orthogonality

• Factorial experimentation

Randomization is a method that protects against an unknown bias distorting the results of the experiment.

An example of a bias is instrument drift in an experiment comparing a baseline procedure to a new procedure. If all the tests using the baseline procedure are conducted first and then all the tests using the new procedure are conducted, the observed difference between the procedures might be entirely due to instrument drift. To guard against erroneous conclusions, the testing sequence of the baseline and new procedures should be in random order such as B, N, N, B, N, B, and so on. The instrument drift or any unknown bias should "average out." Replication increases the sample size and is a method for increasing the precision of the experiment. Replication increases the signal-to-noise ratio when the

noise originates from uncontrollable nuisance variables. A replicate is a complete repetition of the same experimental conditions, beginning with the initial setup. A special design called a Split Plot can be used if some of the factors are hard to vary.

Blocking is a method for increasing precision by removing the effect of known nuisance factors. An example of a known nuisance factor is batch-to-batch variability. In a blocked design, both the baseline and new procedures are applied to samples of material from one batch, then to samples from another batch, and so on. The difference between the new and baseline procedures is not influenced by the batch-to-batch differences. Blocking is a restriction of complete randomization, since both procedures are always applied to each batch. Blocking increases precision since the batch-to-batch variability is removed from the "experimental error.

Orthogonality in an experiment results in the factor effects being uncorrelated and therefore more easily interpreted. The factors in an orthogonal experiment design are varied independently of each other. The main results of data collected using this design can often be summarized by taking differences of averages and can be shown graphically by using simple plots of suitably chosen sets of averages. In these days of powerful computers and software, orthogonality is no longer a necessity, but it is still a desirable property because of the ease of explaining results.

Factorial experimentation is a method in which the effects due to each factor and to combinations of factors are estimated. Factorial designs are geometrically constructed and vary all the factors simultaneously and orthogonally. Factorial designs collect data at the vertices of a cube in p-dimensions (p is the number of factors being studied). If data are collected from all of the vertices, the design is a full factorial, requiring 2^p runs. Since the

total number of combinations increases exponentially with the number of factors studied, fractions of the full factorial design can be constructed. As the number of factors increases, the fractions become smaller and smaller (1/2, 1/4, 1/8, 1/16,..).Fractional factorial designs collect data from a specific subset of all possible vertices and require (2^{p-q}) runs, with 2^{-q} being the fractional size of the design. If there are only three factors in the experiment, the geometry of the experimental design for a full factorial experiment requires eight runs, and a one-half fractional factorial experiment (an inscribed tetrahedron) requires four runs (Fig. 5).

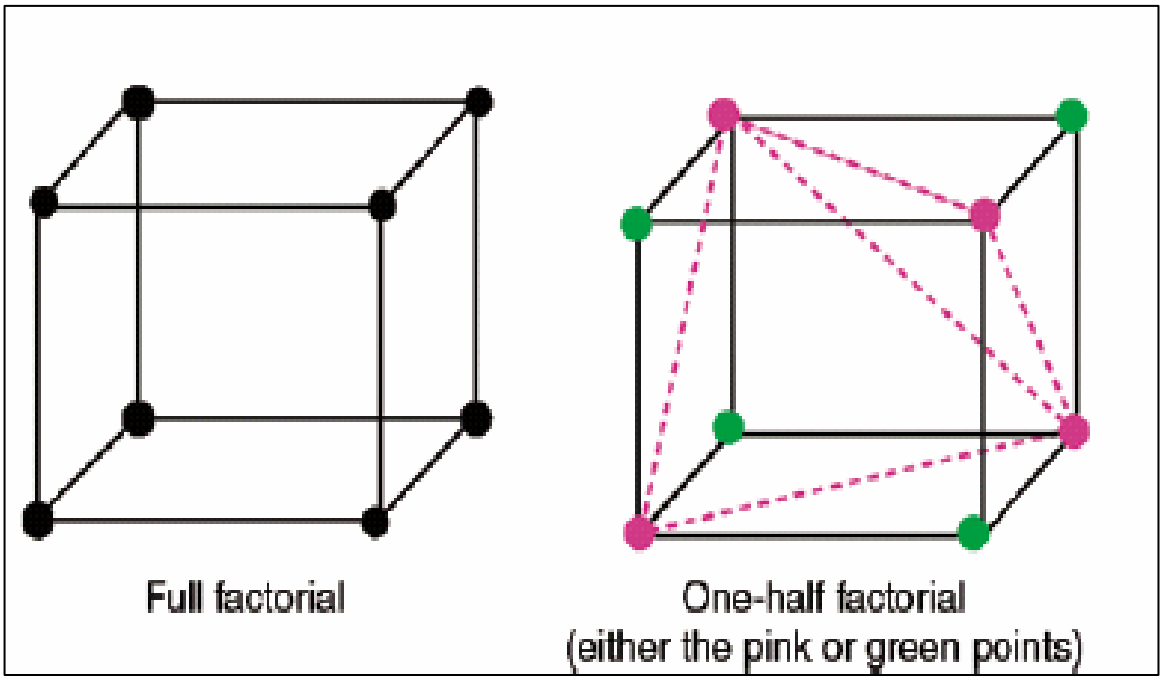


Figure 5 Full factorial and one-half factorial in three dimensions

Factorial designs, including fractional factorials, have increased precision over other types of designs because they have built-in internal replication. Factor effects are essentially the difference between the average of all runs at the two levels for a factor, such as "high" and "low." Replicates of the same points are not needed in a factorial design, which seems like a violation of the replication principle in design of experiments. However, half of all the data points are taken at the high level and the other half are taken at the low level of each factor, resulting in a very large number of replicates. Replication is also provided by the factors included in the design that turn out to have nonsignificant effects. Because each factor is varied with respect to all of the factors, information on all factors is collected by each run. In fact, every data point is used in the analysis many times as well as in the estimation of every effect and interaction. Additional efficiency of the two-level factorial design comes from the fact that it spans the factor space, that is, puts half of the design points at each end of the range, which is the most powerful way of determining whether a factor has a significant effect.

1.4.3.2 Uses

The main uses of design of experiments are

• Discovering interactions among factors

• Screening many factors

• Establishing and maintaining quality control

• Optimizing a process, including evolutionary operations (EVOP)

• Designing robust products

Interaction occurs when the effect on the response of a change in the level of one factor from low to high depends on the level of another factor. In other words, when an interaction is present between two factors, the combined effect of those two factors on the response variable cannot be predicted from the separate effects. The effect of two factors acting in combination can either be greater (synergy) or less (interference) than would be expected from each factor separately.

Frequently there is a need to evaluate a process with many input variables and with measured output variables. This process could be a complex computer simulation model or a manufacturing process with raw materials, temperature, and pressure as the inputs. A screening experiment tells us which input variables (factors) are causing the majority of the variability in the output (responses), i.e., which factors are the "drivers." A screening experiment usually involves only two levels of each factor and can also be called characterization testing or sensitivity analysis.

A process is "out of statistical control" when either the mean or the variability is outside its specifications. When this happens, the cause must be found and corrected. The cause is found efficiently using an experimental design similar to the screening design, except that the number of levels for the factors need not be two for all the factors.

Optimizing a process involves determining the shape of the response variable. Usually a screening design is performed first to find the relatively few important factors. A response surface design has several (usually three or four) levels on each of the factors. This produces a more detailed picture of the surface, especially providing information on which factors have curvature and on areas in the response where peaks and plateaus occur. The EVOP method is an optimization procedure used when only small changes in the

factors can be tolerated in order for normal operations to continue. Examples of EVOP are optimizing the cracking process on crude oil while still running the oil refinery or tuning the welding power of a welding robot in a car manufacturing assembly line.

Product robustness, pioneered by Taguchi, uses experimental design to study the response surfaces associated with both the product means and variances to choose appropriate factor settings so that variance and bias are both small simultaneously. Designing a robust product means learning how to make the response variable insensitive to uncontrollable manufacturing process variability or to the use conditions of the product by the customer.

1.4.3.3 Mathematical Formulation and Terminology

The input variables on the experiment are called factors. The performance measures resulting from the experiment are called responses. Polynomial equations are Taylor series approximations to the unknown true functional form of the response variable. An often quoted insight of George Box is, "All models are wrong. Some are useful." The trick is to have the simplest model that captures the main features of the data or process. The polynomial equation, shown to the third order in Eq. 1 Scheme 1 used to model the response variable *Y* as a function of the input factors X's is

$$Y = \beta_0 + \sum_{i=1}^{p} \beta_i X_i + \sum_{\substack{i=1 \\ i \neq j}}^{p} \sum_{j=1}^{p} \beta_{ij} X_i X_j + \sum_{\substack{i=1 \\ i \neq j \neq k}}^{p} \sum_{j=1}^{p} \sum_{k=1}^{p} \beta_{ijk} X_i X_j X_k + \cdots, \quad (1)$$

Scheme 1: The polynomial equation used to model the response variable *Y* as a function of the input factors X's where:

β_0 = the overall mean response,

β_I = the main effect for factor (i = 1, 2, ... , p),

β_{ij} = the two-way interaction between the *i*th and *j*th factors, and

β_{ijk} = the three-way interaction between the *i*th, *j*th, and *k*th factors.

Usually, two values (called levels) of the X's are used in the experiment for each factor, denoted by high and low and coded as 11 and 21, respectively. A general recommendation for setting the factor ranges is to set the levels far enough apart so that one would expect to see a difference in the response but not so far apart as to be out of the likely operating range. The use of only two levels seems to imply that the effects must be linear, but the assumption of monotonicity (or nearly so) on the response variable is sufficient. At least three levels of the factors would be required to detect curvature.

Interaction is present when the effect of a factor on the response variable depends on the setting level of another factor. Graphically, this can be seen as two nonparallel lines when plotting the averages from the four combinations of high and low levels of the two factors. The βij terms in Eq. 1 account for the two-way interactions. Two-way interactions can be thought of as the corrections to a model of simple additivity of the factor effects, the model with only the βI terms in Eq. 1. The use of the simple additive model assumes that the factors act separately and independently on the response variable, which is not a very reasonable assumption.

Experimental designs can be categorized by their resolution level. A design with a higher resolution level can fit higher-order terms in Eq. 1 than a design with a lower resolution level. If a high enough resolution level design is not used, only the linear combination of several terms can be estimated, not the terms separately. The word "resolution" was borrowed from the term used in optics. Resolution levels are usually denoted by Roman numerals, with III, IV, and V being the most commonly used. To resolve all of the two-way interactions, the resolution level must be at least V. Four resolution levels and their meanings are given in Table 2.

Table 2 Resolution levels and their meanings

Resolution level	Meaning
II	Main effects are linearly combined with each other ($\beta_i + \beta_j$).
III	Main effects are linearly combined with two-way interactions ($\beta_i + \beta_{jk}$).
IV	Main effects are linearly combined with three-way interactions ($\beta_i + \beta_{jkl}$) and two-way interactions with each other ($\beta_{ij} + \beta_{kl}$).
V	Main effects and two-way interactions are not linearly combined except with higher-order interactions ($\beta_i + \beta_{jklm}$ and $\beta_{ij} + \beta_{klm}$).

1.4.3.4 Number of Runs need for Factorial Experimental Design

Many factors can be used in a screening experiment for a sensitivity analysis to determine which factors are the main drivers of the response variable. However, as noted earlier, as the number of factors increases, the total number of combinations increases exponentially. Thus, screening studies often use a fractional factorial design, which produces high confidence in the sensitivity results using a feasible number of runs.

Fractional factorial designs yield polynomial equations approximating the true response function, with better approximations from higher resolution level designs. The minimum number of runs needed for Resolution IV and V designs is shown in Table 3 as a function of the number of factors in the experiment.

There is a simple relationship for the minimum number of runs needed for a Resolution IV design: round up the number of factors to a power of two and then multiply by two. The usefulness of Table 3 is to show that often there is no penalty for including more factors in the experiment. For example, if 33 factors are going to be studied already, then up to 64 factors can be studied for the same number of runs, namely, 128. It is more desirable to conduct a Resolution V experiment to be able to estimate separately all the two-way interactions. However, for a large number of factors, it may not be feasible to perform the Resolution V design. Because the significant two-way interactions are most likely to be combinations of the significant main effects, a Resolution IV design can be used first, especially if it is known that the factors have monotonic effects on the response variable. Then a follow-up Resolution V design can be performed to determine if there are any significant two-way interactions using only the factors found to have significant

effects from the Resolution IV experiment. If a factorial design is used as the screening experiment on many factors, the same combinations of factors need not be replicated, even if the simulation is stochastic. Different design points are preferable to replicating the same points since more effects can be estimated, possibly up to the next higher resolution level.

Table3. Two-level designs: minimum number of runs as a function of number of factors

Factors	Runs
Resolution IV	
1	2
2	$4 = 2^2$
3–4	$8 = 2^3$
5–8	$16 = 2^4$
9–16	$32 = 2^5$
17–32	$64 = 2^6$
33–64	$128 = 2^7$
65–128	$256 = 2^8$
129–256	$512 = 2^9$
Resolution V	
1	2
2	$4 = 2^2$
3	$8 = 2^3$
4–5	$16 = 2^4$
6	$32 = 2^5$
7–8	$64 = 2^6$
9–11	$128 = 2^7$
12–17	$256 = 2^8$
18–22	$512 = 2^9$
23–31	$1,024 = 2^{10}$
32–40	$2,048 = 2^{11}$
41–54	$4,096 = 2^{12}$
55–70	$8,192 = 2^{13}$
71–93	$16,394 = 2^{14}$
94–119	$32,768 = 2^{15}$

1.4.3.5 Implementation

The main steps to implement an experimental design are as follows. Note that the subject matter experts are the main contributors to the most important steps, i.e., 1–4, 10, and 12.

1. State the objective of the study and the hypotheses to be tested.
2. Determine the response variable(s) of interest that can be measured.
3. Determine the controllable factors of interest that might affect the response variables and the levels of each factor to be used in the experiment. It is better to include more factors in the design than to exclude factors, that is, prejudging them to be nonsignificant.
4. Determine the uncontrollable variables that might affect the response variables, blocking the known nuisance variables and randomizing the runs to protect against unknown nuisance variables.
5. Determine the total number of runs in the experiment, ideally using estimates of variability, precision required, size of effects expected, etc., but more likely based on available time and resources. Reserve some resources for unforeseen contingencies and follow-up runs. Some practitioners recommend using only 25% of the resources in the first experiment.
6. Design the experiment, remembering to randomize the runs.
7. Perform a pro forma analysis with response variables as random variables to check for estimability of the factor effects and precision of the experiment.

8. Perform the experiment strictly according to the experimental design, including the initial setup for each run in a physical experiment. Do not swap the run order to make the job easier.
9. Analyze the data from the experiment using the analysis of variance method developed by Fisher.
10. Interpret the results and state the conclusions in terms of the subject matter.
11. Consider performing a second, confirmatory experiment if the conclusions are very important or are likely to be controversial.
12. Document and summarize the results and conclusions, in tabular and graphical form, for the report or presentation on the study. [42]

1.4.4 Simplex Optimization

The simplex method is based on an initial design of k+1 trials, where k is the number of variables. A k+1 geometric figure in a k-dimensional space is called a simplex. The corners of this figure are called vertices. A simplex is a geometric figure having one more vertex than its number of factors. Therefore a simplex in one dimension is a line, in two dimensions a triangle, in three dimensions a tetrahedron, see figure 6, and in multiple dimensions a hyper-tetrahedron. A geometric interpretation is difficult with more variables, but the basic mathematical approach outlined below can handle the search for optimum conditions (Otto, 1999).

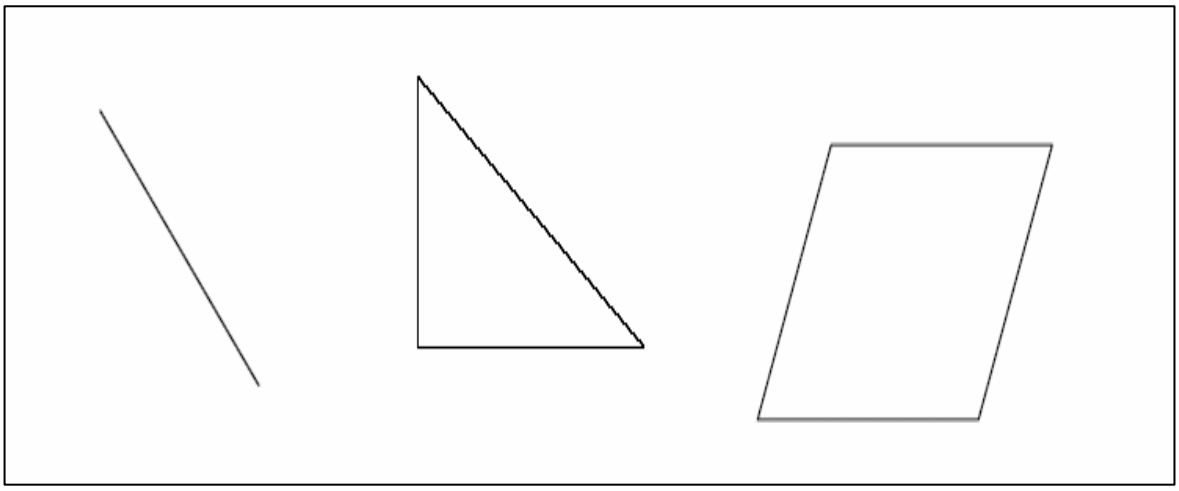


Figure 6 Geometric interpretation of lower dimensional simplex. The shapes of the simplex in a one, a two and a three variable search space, are a line, a triangle or a tetrahedron.

1.4.4.1 The basic simplex method

The basic simplex method is easy to understand and apply. The optimization begins with the initial trials. The trial conditions are spread out efficiently. These initial trials form the first simplex. The number of initial trials is equal to the number of control variables plus one. With two variables, the first simple design is based on three trials for three variables it is four trials, etc. This number of trials is also the minimum for defining a direction of improvement. Therefore, it is a time saving and economical way to start an optimization project. After the initial trials, the simplex process is sequential, with the addition and evaluation of one new trial at a time. The simplex searches systematically for the best levels of the control variables. The optimization process ends when the optimization objective is reached or when the responses cannot be further improved, see figure 7.

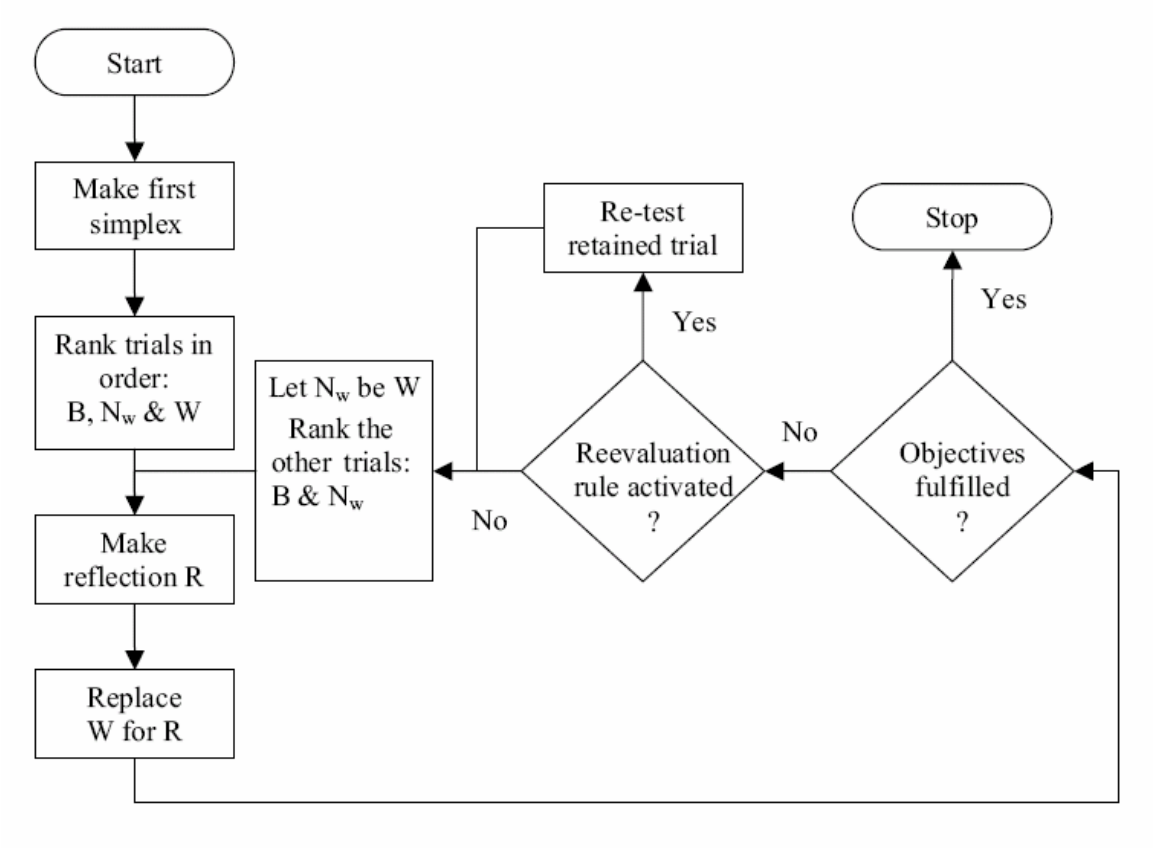


Figure 7 Basic simplex rules

The calculations in the basic simplex algorithm are outlined in the flow chart. For each simplex, the following labels are used: W for the least favorable trial or the trial being rejected, B for the most favorable trial and N_w FOR the second least favorable trial (i.e. next to worst).

The first rule is to reject the trial with the least favourable response value in the current simplex. A new set of control variable levels is calculated, by reflection into the control variable space opposite the undesirable result. This new trial (R) replaces the least favourable trial in the next simplex. This leads to a new least favourable response in the simplex that, in turn, leads to another new trial, and so on. Through this, the simplex will move steadily towards more favourable conditions away from the least favourable conditions

These steps are repeated until the simplex begins to rotate around the optimum or the response satisfies the experimenters´ needs (1996; Otto, 1999), se figure 8. The fixed step width of the fixed size simplex might lead to problems if the step width chosen is either too large or too small. In the first case, the optimum might be missed and in the latter the number of experiments required becomes very large. These disadvantages can be circumvented if the step width is tuneable, as with in the variable-size simplex or modified simplex method (Otto, 1999).

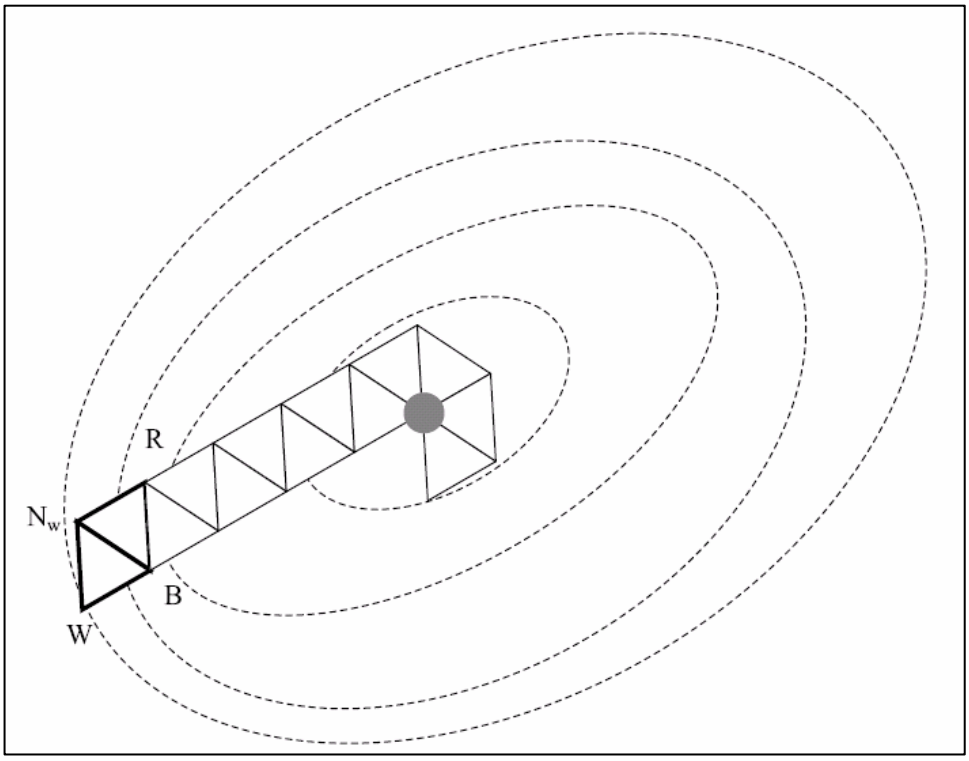


Figure 8 Illustration of a simplex path with two variables. After three initial trials the corners of the triangle are classified as W for the least favorable trial or the trial being rejected, B for the most favorable trial and Nw for the second least favorable trial (i.e. next to worst). Then the next trial 4 (R) is calculated and performed.

1.4.4.2 The modified simplex method

The modified simplex method has much in common with the basic method, but can adjust its shape and size depending on the response in each step. Several new rules are added to the basic simplex rules. These rules make the simplex expand, in a direction of more favourable conditions, or contract if a move was taken in a direction of less favourable conditions.

The procedure for expansion and contraction enable the modified simplex both to accelerate along a successful track of improvement and to home in on the optimum conditions. Therefore the modified simplex will usually reach the optimum region quicker than the basic method will and will pinpoint the optimum levels more closely. Moves in a typical optimization sequence are easy to show for control variables (the response is shown separately).

The degree of contraction depends on how unfavourable the new response is. With the modified simplex, the step width is changed by expansion (E) and contraction (C- and C+) of the reflected vertices, see figures 9 and 10. [43]

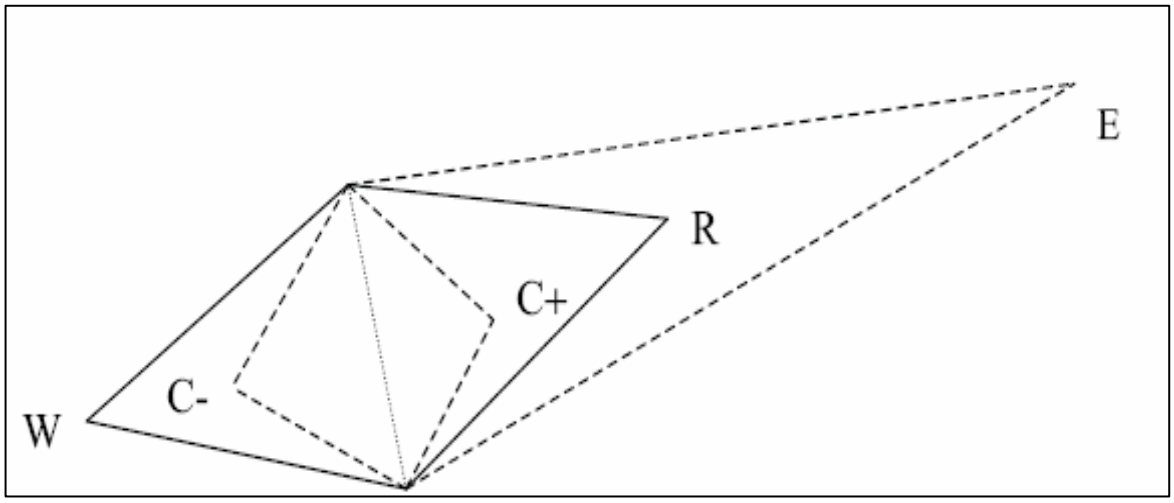


Figure 9 Illustration of the different moves with the modified simplex method, adding the expansion and contraction of the reflected vertices

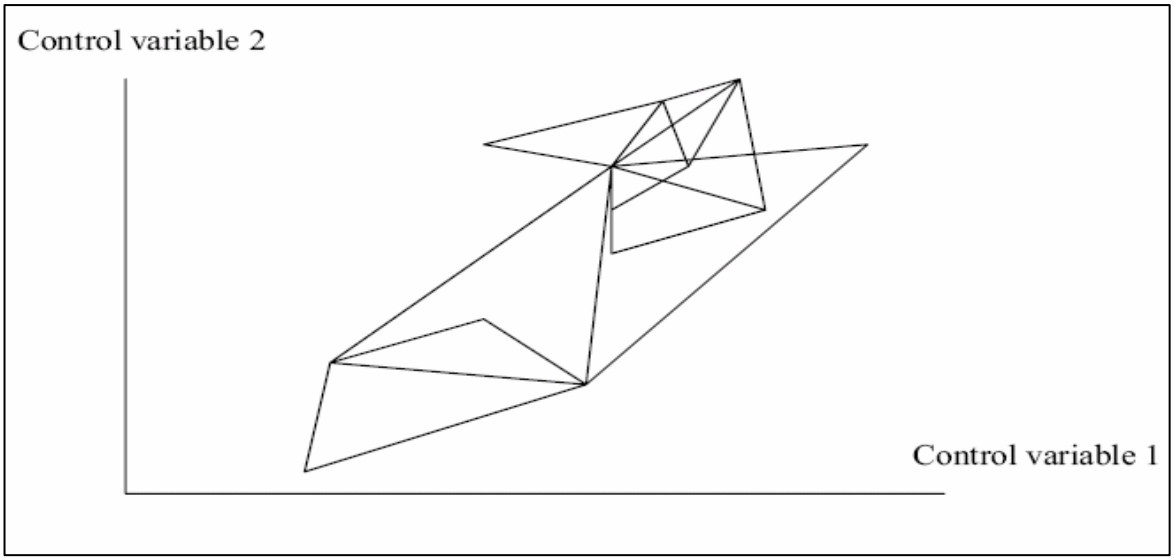


Figure 10 An example of a typical optimization sequence with modified simplex method. Change in the levels for two control variables.

1.4.4.3 MultiSimplex

The MultiSimplex program is designed as a true multivariate non-linear optimization tool that combines the modified simplex method [44] with the fuzzy set theory [45] by means of the membership functions or the point response measure called the aggregated value or membership. The advantage of MultiSimplex is that it allows simultaneous optimization of several response signals and considers the interaction among variables. MultiSimplex optimization is easy to follow and has been already used in some analytical application. To do the MultiSimplex optimization, the variables, the range of each variable and the responses that are going to be followed are defined. Then MultiSimplex suggests a $K + 1$ number of experiments; where K is the number of variables to be studied. Once the experiments are carried out, the answers of the experiments are introduced and MultiSimplex suggests one new experiment. The process is continued until the optimum conditions are reached. In order to measure the closeness to the optimum, MultiSimplex makes use of the "membership value" [44]. This value ranges from 0 to l and takes into account the results of all responses considered in the optimization. Optimized conditions are achieved when the membership value is close to 1. The optimization procedure includes a re-evaluation rule that means, for every certain number of experiments, a previous trial is repeated experimentally.

1.5 METHODS VALIDATION

The FDA in its most recent publication, *Guidance for Industry on Analytical Procedures and Methods Validation*, states:

Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures. [46]

What the FDA does not say is that the actual validation component of the methods validation process should be the culmination of a well-organized, well-planned, and systematically executed process that includes method development, prevalidation studies, and finally, methods validation itself. Gone are the days where one did methods development/validation concurrently. Validation is the end game where few surprises and deviations are expected. Validation is executed with a formal, approved and signed methods validation protocol in place which has been reviewed by the quality assurance (QA) unit. Validation is complete when you:

(1) Demonstrate that you have met all the acceptance criteria.

(2) Clearly document the results in a CGMP compliant fashion.

(3) Show how you met the acceptance criteria in a final methods validation report, including references to raw data, all of which have been reviewed and approved by the appropriate personnel including peers, management, and QA.

Some would even argue that the validation process is not complete until the methods are successfully transferred to their end-user laboratories.

This sounds like a daunting task. And to be completely honest—it is. There is nothing trivial or easy about methods validation. It takes time, resources, and rarely goes as easy as you think it's going to go. Methods validation is part science, part art, and a lot of bookkeeping and accounting. To be brutally honest, too few laboratories do a very good job executing all the components.

Due to the magnitude of the task, the time, and the perceived costs, many laboratories try to cut corners. At a minimum, this results in deviations from the protocol which no longer can be "arm waved" away. The FDA expects you to scientifically address failures as you would any other laboratory investigation. This takes more time and effort and often results in delays in the validation timeline. In the worst case, you end up validating a method that is transferred to quality control (QC) labs worldwide, and ends up being the root cause of untold laboratory investigations. It is hypothesized that many of the problems discovered during root-cause analysis of out-of-specification results (OOS) are a direct result of poorly or partially validated methods.

1.5.1 Approach

The following are typical analytical performance characteristics which may be tested during methods validation:

- Accuracy
- Precision
    - Repeatability and Intermediate precision
- Specificity

- Detection limit
- Quantitation limit
- Linearity
- Range
- Robustness
- System suitability determination
- Forced degradation studies
- Solution stability studies
- Filter retention studies
- Extraction efficiency studies
- Additional methods validation information
    - Representative instrument output
    - Representative calculations
    - Listing and characterization of known impurities
    - Degradation pathways (if known)

A more detailed definition of some characteristic is given in the following subsections.

1.5.1.1 Accuracy

Accuracy is the nearness of a measured value to the true or accepted value. It provides an indication of any systematic error or bias in the method. For an unbiased method, a theoretical plot of measured value versus true value can be described by a mathematical function. In the pharmaceutical industry, this is typically a straight line with a given slope

and zero intercept. It follows that the accuracy of a biased method varies with the analyte concentration according to the types of systematic errors.

During the validation, accuracy is determined by measuring the recovery of the active component from a drug product matrix or by directly measuring the active pharmaceutical ingredient (API). Typically studies involve spiking the drug product placebo matrix with API in amounts equal to the nominal finished dosage strength. This spiking is either by adding of standard solutions or dry spiking API into the matrix followed by complete mixing.

1.5.1.2 Precision

Precision consists of two components: repeatability and intermediate precision. Repeatability is the variation experienced by a single analyst on a single instrument. Repeatability does not distinguish between variation from the instrument or system alone and from the sample preparation process. During the validation, repeatability is performed by analyzing multiple replicates of an assay composite sample by using the analytical method. The recovery value is calculated and reported for each value.

Intermediate precision refers to variations within a laboratory such as different days, with different instruments, and by different analysts. This was formerly known as ruggedness. During the validation, a second analyst repeats the repeatability analysis on a different day using different conditions and different instruments. Recovery values are calculated and reported. A statistical comparison is made to the first analyst's results.

1.5.1.3 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products, and excipients. There must be inarguable data for a method to be specific. Specificity = measures only the desired component without interference from other species that might be present; separation is not necessarily required.

To determine specificity during the validation blanks, sample matrix (placebo), and known related impurities are analyzed to determine whether interferences occur. Specificity is also demonstrated during forced degradation studies.

The term "selectivity" is sometimes used interchangeably with specificity. Technically, however, there is a difference. Selectivity is defined as the ability of the method to separate the analyte from other components that may be present in the sample, including impurities. Selectivity is separate and shows every component in the sample. Therefore, one could have a method that is specific, yet it may not be selective. For instance, an ion selective electrode may be specific (e.g., is used to measure a single species in sample matrix), yet not be selective (e.g., doesn't separate and identify all components present).

1.5.1.4 Detection Limit

The detection limit (DL) or limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The LOD is a parameter of limit tests (i.e., tests that only determine if the analyte concentration is above or below a specification limit).

In analytical procedures such as HPLC that exhibit baseline noise, the LOD can be based on a signal-to-noise (S/N) ratio (3:1), which is usually expressed as the concentration (e.g., percentage, parts per billion) of analyte in the sample. There are several ways in which it can be determined, but it usually involves injecting samples, which generate an S/N of 3:1, and estimating the DL.

1.5.1.5 Quantitation Limit

The quantitation limit (QL) or limit of quantitation (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low concentrations of compounds in sample matrices and is used particularly for the determination of impurities and/or degradation products. It is usually expressed as the concentration (e.g., percentage, parts per million) of analyte in the sample.

For analytical procedures such as HPLC that exhibit baseline noise, the LOQ is generally estimated from a determination of S/N ratio (10:1) and is usually confirmed by injecting standards which give this S/N ratio and have an acceptable percent relative standard deviations (%RSDs) as well.

1.5.1.6 Linearity

Linearity evaluates the analytical procedure's ability (within a give range) to obtain a response that is directly proportional to the concentration (amount) of analyte in the

sample. If the method is linear, the test results are directly or by well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range. Linearity is usually expressed as the confidence limit around the slope of the regression line. The line is calculated according to an established mathematical relationship from the test response obtained by the analysis of samples with varying concentrations of analyte. Note that this is different from *range* (sometimes referred to as *linearity of method*) which is evaluated using samples and must encompass the specification range of the component assayed in the drug product.

During validation, linearity may be established for all active substances, preservatives, and expected impurities. Evaluation is usually performed on standards.

1.5.1.7 Range

Range is defined as the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity.

Range is normally expressed in the same units as test results (e.g., percent, parts per million) obtained by the analytical method.

During validation, range (sometimes referred to as *linearity of method*) is evaluated using samples (usually spiked placebos) and must encompass the specification range of the component assayed in the drug product.

1.5.1.8 Robustness

Robustness is defined as the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g., pH, mobile-phase composition, temperature, and instrument settings) and provides an indication of its reliability during normal usage. This is an important parameter with respect to the transferability of the method following validation.

Determining robustness is a systematic process of varying a parameter and measuring the effect on the method by monitoring system suitability and/or the analysis of samples. It is part of the formal methods validation process. [47]

CHAPTER 2

EXPERIMENTAL

The ultimate objective of this research is to develop automated SIA methods that can be applied directly in the pharmaceutical industry therefore, it's highly imperative that the chemistry of the proposed methods be aligned with already published chemistry for the drug analysis of Ketoconazole and Diclofenac sodium. The computer flow programs for these SIA methods were written, optimized using the univariate method of approach, empirical approach and chemometrics optimization using the MultiSimplex® for Windows version 2.1.3 (2001) supplied by Grabitech Solutions AB (Publ), Sundsvall, Sweden for chemometric optimization

2.1 CHEMICAL AND REAGENTS PREPARATIONS

The reagents and solution for the adapted SIA methods were prepared using analar grade chemicals according to the method procedure. Distilled water used was double distilled and deionized.

2.2 INSTRUMENTATION

The manifold to be used in this method consists of SIA combined with a fiber optic spectrometer. The SIA system is the *FIALab 3500* (Medina, WA USA). It is composed of a syringe pump, a multi-position valve, a Z-flow cell with SMA fiber optic connectors as well as pump tubing and PC. The *Syringe Pump* is 24,000 steps with an optical encoder feedback and 1.5 seconds to 20 minutes per stroke of 2.5 ml size. It is > 99% accuracy at full stroke. The volume capacity of syringe is 2500 µl. The *Multi-Position Valve* has eight ports with a standard pressure of 250 psi (gas)/600 psi (liquid); zero dead volume; chemically inert; port selection is usually done using the software program. The Z-Flow *Cell* is 10 mm path length plexiglass compatible with standard SMA terminated fiber optics was used. *Pump Tubing* of 0.30" ID Teflon type supplied by Upchurch Scientific, Inc. (Oak Harbor, WA, USA) was used for connecting the different units; and making both the holding coil (190 cm long) and the reaction coil (190 cm long). The fiber optic spectrometer is composed of a light source, 200 micron fiber optic connectors and a detector. The light source is *LS-1 Tungsten Halogen* (Ocean Optics, USA) optimized for VIS-NIR (360nm-2µm) wavelength range. The detector is *USB2000 Spectrometer* (Ocean Optics, USA) adapted to 200-1100 nm wavelength range. The SIA manifold is illustrated below Fig 11 and Fig 12.

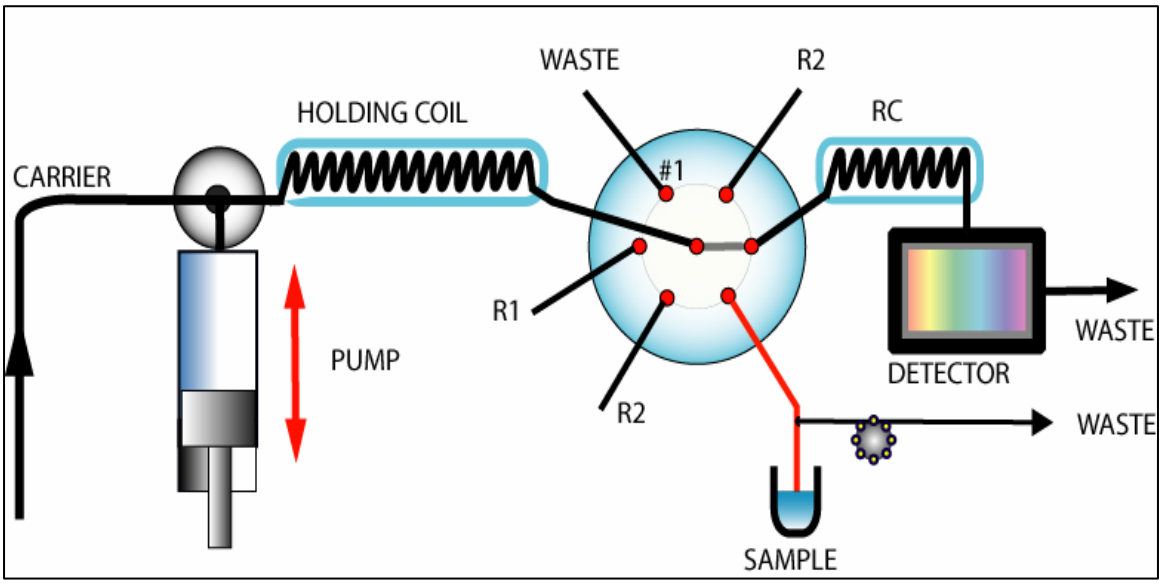


Figure11. Schematic of SIA manifold used for the proposed SIA methods for Ketoconazole, Diclofenac determination.

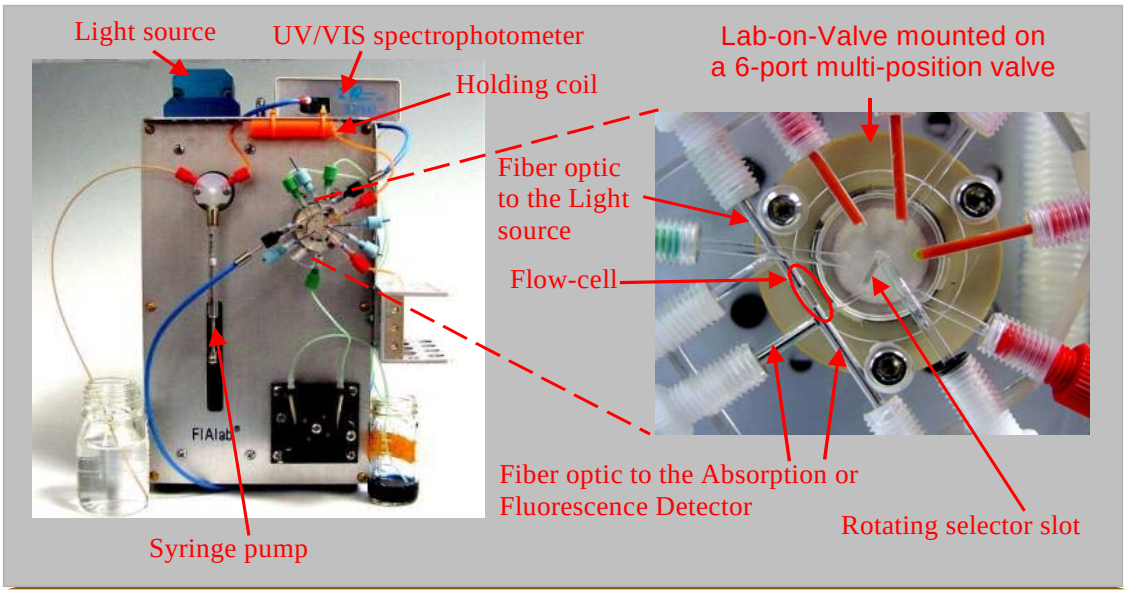


Figure 12: SIA instrumentation showing the channeled multiselection valve, fiber optic Cables for light source and computer processor

2.3 SOFTWARE PACKAGE

FIALab® for Windows version 5.0 from FIAlab Co. (Medina, WA USA) for programming and controlling the SI analyzer manifold. OOIBase® version 2.0.1.2, driver version 4.07.00 (2002), supplied from Ocean Optic, Inc for acquisition and treatment of the spectrophotometric data

SigmaPlot® for Windows version 9.01 from Systat Software, Inc. for data interpolation and constructing surface plots.

MultiSimplex® for Windows version 2.1.3 (2001) supplied by Grabitech Solutions AB (Publ), Sundsvall, Sweden for chemometric optimization.

SigmaStat® supplied from Jandel Scientific Corporation (93/ 1994) for chemometric calculations. SPSS® for Windows version 10.0.1 for statistical analysis.

Minitab software for analysis of variance (ANOVA)

2.4 THE SEQUENTIAL STEPS FOR ANALYSIS

The procedure steps followed for the assay of ketoconazole and diclofenac in pharmaceutical preparations is described as under utilizing the Alitea FIALab software for controlling different SIA components. Similar programs for preliminary investigations and optimization were designed similarly as shown in Appendices I to III.

I. Water will be linked to the SP through the in-position mode to push reagents to the required part of the SI analyzer manifold.

II. Standard solutions will be linked to the MPV through ports 2, 3, 4 and 5.

III. The syringe will fill with 5000 µl of water by directing the two-way valve to the in-position mode with flow rate of 50µl/s

IV. Tubes will be loaded with their respective reagents by performing aspiration runs and directing the two-way valve to the out-position mode with flow rate of 50µl/s

V. With a different flow rate, the syringe will be dispensing 2000 µl.

VI. The appropriate volume of the drugs (Ketoconazole or Diclofenac) and cerium (IV) in µl will be sequentially aspirated into the HC.

VII. The appropriate volume of product will be dispensed at the required flow rate to the Z and, simultaneously, the reference and absorbance scan will be carried out. Maximum value of the monitor peak will record as the value of the absorbance of the drug solution

2.5 SIA INSTRUMENT VALIDATION

Validation of the SIA system was carried out by conducting the Operation Qualification (OQ) using potassium permanganate as a stable well known standard. The Operation Qualification (OQ) was conducted to trust that all data generated is precise and accurate.

2.5.1 Preparation of Permanganate Standard Solutions

A standard solution was prepared by dissolving exactly about 3.5 g of dried potassium permanganate (P-279 Lot 746030 Fisher scientific company, USA) in 1000 ml with acidified water of pH 6.0. The stock solution was standardized with sodium oxalate (S-356 Lot 792406 Fisher scientific company, USA). This solution was used through all the experimental processes.

2.5.2 Method and Procedure

The full text of the computer program used for the calibration of SIA system can be found in the appendice I (page 163); the full components of the SIA manifold are diagramed in fig. 13. The following steps were the protocol applied for the calibration:

I. Water was linked to the SP through the in-position mode to push reagents to the required part of the SI analyzer manifold.

II. $KMnO_4$ Standard solutions were linked to the MPV through ports 2, 3, 4, 5, and 6

III. The syringe was filled with 5000 µl of water by directing the two-way valve to the in-position mode with flow rate of 100µl/s

IV. Tubes were loaded with their respective solutions by performing aspiration runs and directing the two-way valve to the out-position mode with flow rate of 150µl/s

V. With a different flow rate, the syringe was dispensed 900 µl.

VI. The appropriate volume of potassium standard solutions was aspirated with flow rate of 150µl/s into the HC

VII. The appropriate volume of product will be dispensed at the required flow rate to the Z and, simultaneously, the reference and absorbance scan will be carried out. Maximum value of the monitor peak will record as the value of the absorbance of the appropriate standard solution.

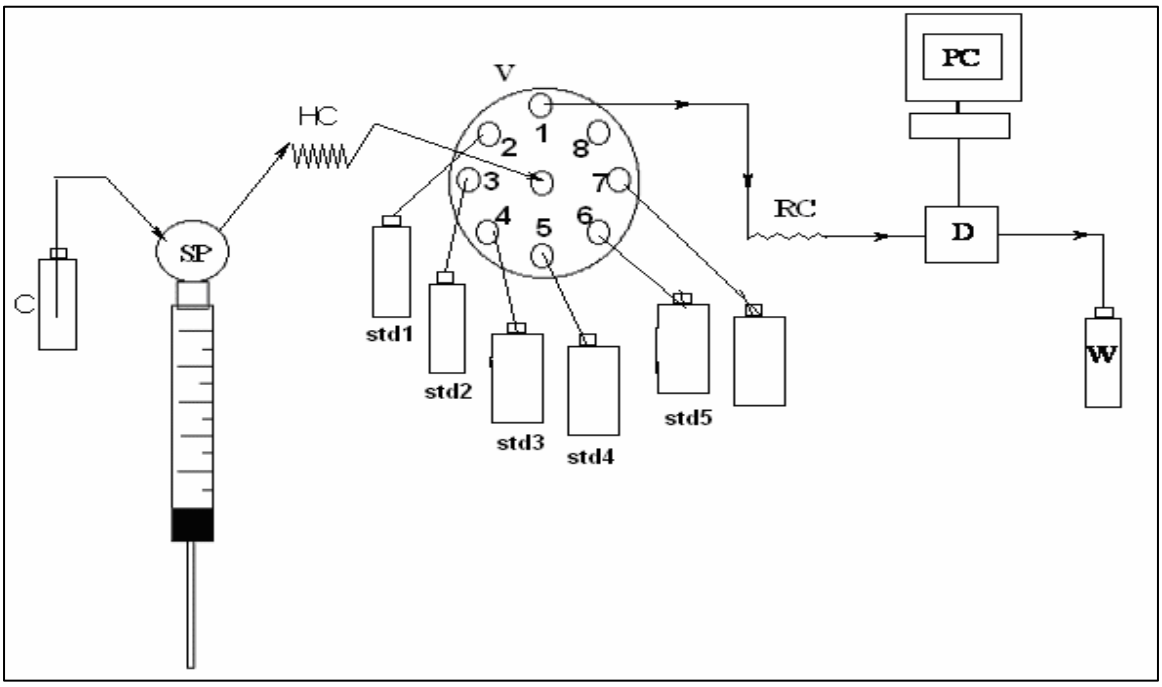


Figure 13: SIA manifold comprised of; A. carrier (water); SP. 5 mL syringe pump; HC. Holding coil; V. eight ports selector valve; RC. Reaction coil; (std1, std2, std3, std4, and std5) = $KMnO_4$ standard solutions; RC. Reaction coil; D. spectrophotometer; PC Computer and W. waste

2.5.3 Calibration Curve

A calibration plot was constructed by running the system at a fixed wavelength of 527 nm using different concentrations of potassium permanganate in the range of 50 to 250 mg l^{-1}. A typical SIA-Gram of absorbance versus concentration is shown in Fig. 14. It is clear that prominent well-defined five peaks are produced with a clear increase in the absorbance maxima; the sixth peak appears for the permanganate solution labeled as an unknown. The absorbances obtained in Fig. 14 were plotted versus the corresponding concentration were linear (Fig. 15) with the following calibration equation: $A = 0.0590 + 0.0109 \times C$; with $R^2 = 0.9985$ indicating excellent linearity with a standard deviation of less than 1.0, emphasizing excellent precision.

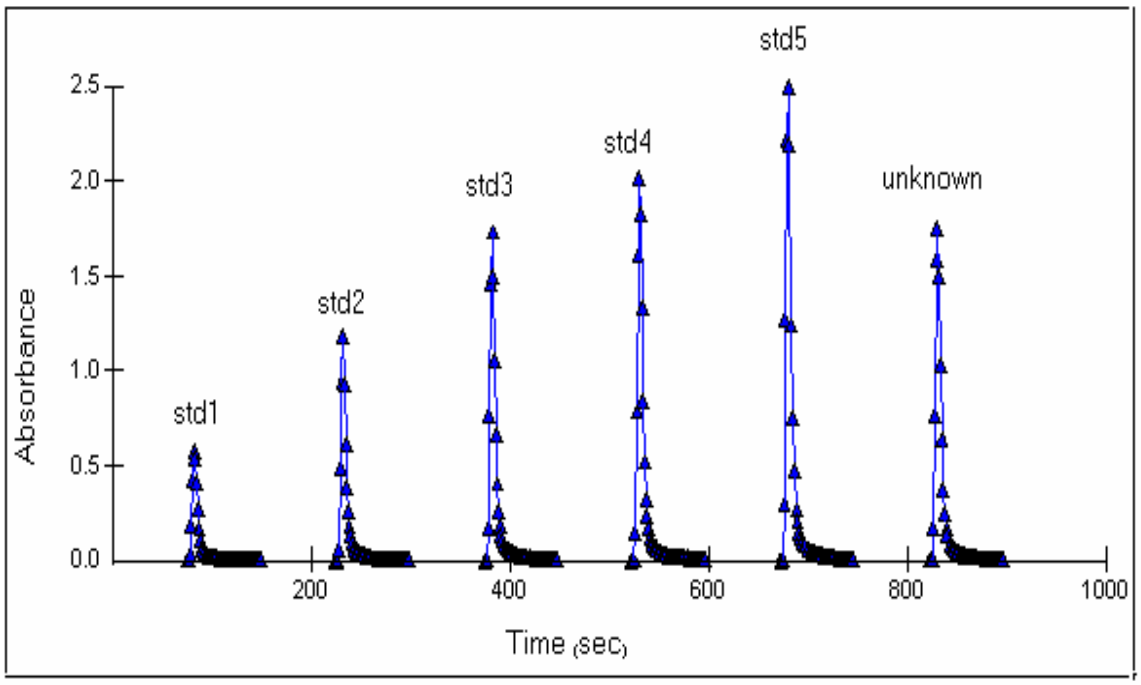


Figure 14 Typical SIA-Gram of Absorbance versus time for different standard concentrations of potassium permanganate as std-1=50 mg l^{-1}; std-2 = 100 mg l^{-1}; std-3 = 150; std-4= 200 mg l^{-1}; std-5 = 250 mg l^{-1}; and an unknown claimed as 150 mg l^{-1}.

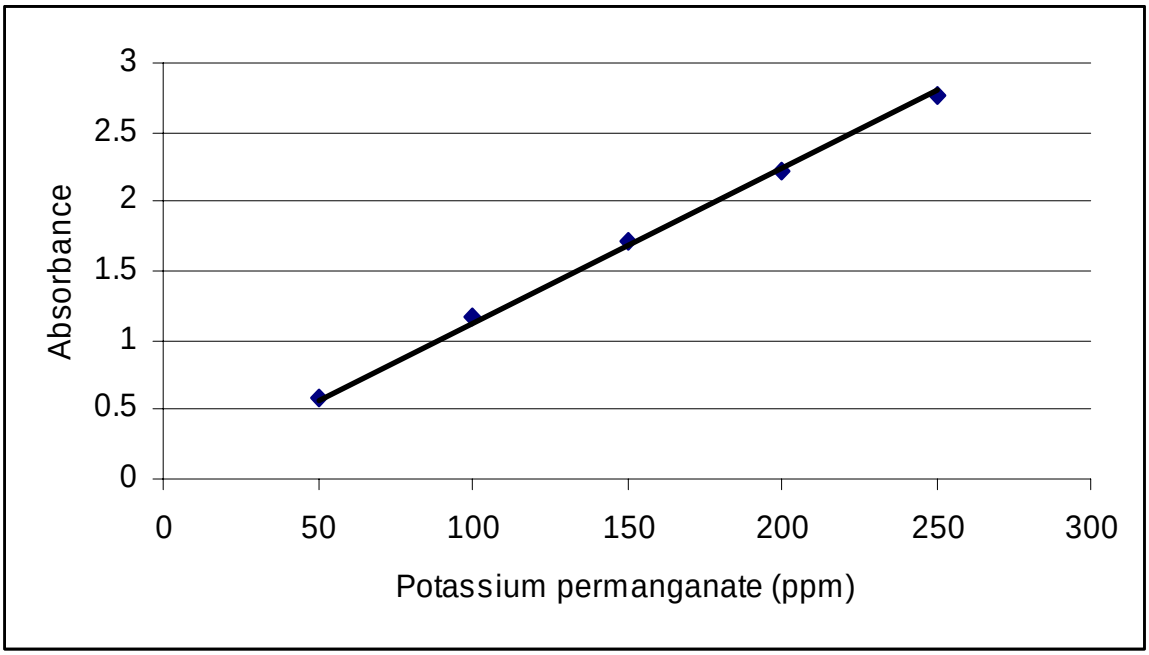


Figure15 Calibration curve utilizing different standard concentrations of potassium permanganate between 50 mg l^{-1} to 250 mg l^{-1}

2.6 DETERMINATION OF KETOCONAZOLE

2.6.1 Preparation of Reagent and Standard Sample Solutions

1. Ketoconazole tablets: 20 tablets from the proprietary drugs were accurately weighed, crushed and powdered. The amount of the powder containing the appropriate weight to give 1000 ppm Ketoconazole was dissolved in about 40 ml of acidified water; heated for three minutes filtered and then it was made up to 100 ml volume in a 100 ml volumetric flask after cooling. Further dilutions could be made from the same.

2. Sulfuric acid Solution (0.01 mol dm^{-3}) A stock solution of 0.01 mol dm^{-3} H_2SO_4 (95-98% Specific gravity 1.84 kg / l, Merck, UK) was prepared the usual way. Working solutions were prepared by dilutions.

3. Cerium (IV) (500 ppm) A stock solution was prepared by dissolving exactly about 0.25 g of dried cerium(IV) ammonium sulfate [Ce(NH4)4SO4.XH2O] (Fluka AG, CH-9470 Buchs, Switzerland) in 10 mL of 0.5 mol L^{-1} sulfuric acid and diluting to 500 ml with distilled water in a calibrated flask.

2.6.2 Method and Procedure

The full text of the computer program used for the determination of ketoconazole can be found in the appendice II (page 171): the full components of the SIA manifold are diagramed in fig. 16. The following steps were the protocol applied for the calibration:

I. Water was linked to the SP through the in-position mode to push reagents to the required part of the SI analyzer manifold.

II. Cerium (IV) (R) was linked to the selector valve through port 2; Ketoconazole standard solutions prepared in the range 5 to 240 ppm were linked to the selector valve through ports 3, 4, 5, 6, and 7 while the unknown concentration of KC which is usually the tablet solution was linked to the selector valve through port 8. Tubings were loaded with their respective reagents by an aspiration run.

III. The syringe was filled with 5000 µl of water by directing the two-way valve to the in-position mode with flow rate of 100µl/s

IV. Tubes were loaded with their respective solutions by performing aspiration runs and directing the two-way valve to the out-position mode with flow rate of 100µl/s

V. With a different flow rate, the syringe was dispensed 2000 µl.

VI. The appropriate volume of Cerium (IV) standard solutions was aspirated with flow rate of 100µl/s into the HC

VII. The appropriate volume of product will be dispensed at the required flow rate to the Z and, simultaneously, the reference and absorbance scan will be carried out. Maximum

value of the monitor peak will record as the value of the absorbance of the appropriate standard solution.

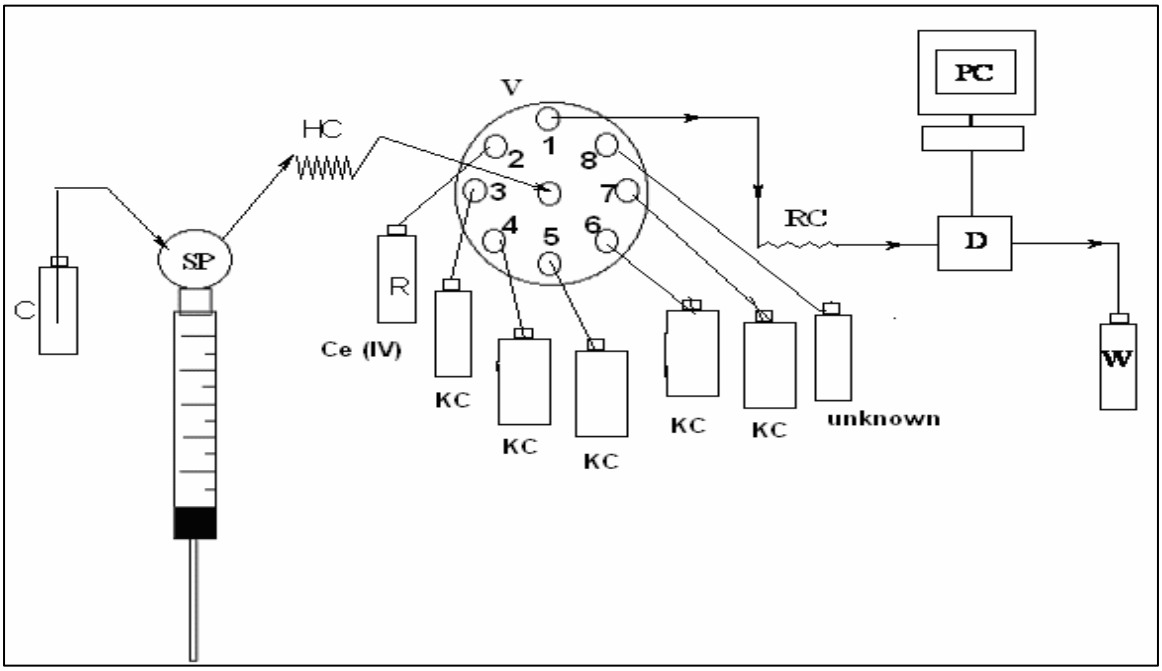


Figure 16: SIA manifold comprised of; A. carrier (water); SP. 5 mL syringe pump; HC. Holding coil; V. eight ports selector valve; RC. Reaction coil ; R Ce (IV); KC Ketoconazole solutions; RC. Reaction coil; D Spectrophotometer; PC Computer and W. waste

2.7 DETERMINATION OF DICLOFENAC SODIUM

2.7.1 Preparation of Reagent and Standard Sample Solutions

1. Diclofenac sodium tablets: 20 tablets from the proprietary drugs were accurately weighed, crushed and powdered. The amount of the powder containing the appropriate weight to give 150 ppm Diclofenac sodium was dissolved in about 40 ml of acidified water; heated for 3 minutes then it was made up to volume in a 100 ml volumetric flask after cooling. Further dilutions could be made from the same.

2. Potassium permanganate (0.0199 mol dm^{-3}): A standard solution was prepared by dissolving exactly about 3.5 g of dried potassium permanganate (P-279 Lot 746030 Fisher scientific company, USA) in 1000 ml with acidified water of pH 6.0. The stock solution was standardized with sodium oxalate (S-356 Lot 792406 Fisher scientific company, USA). This solution was used through all the experimental processes.

3. Sulfuric acid Solution (6.0×10^{-6} mol dm^{-3}). A stock solution of 6.0×10^{-6} mol dm^{-3} H_2SO_4 (95-98% Specific gravity 1.84 kg / l, Merck, UK) was prepared the usual way. Working solutions were prepared by dilutions.

2.7.2 Method and Procedure

The full text of the computer program used for the determination of Diclofenac sodium (DCS) can be found in the appendice III (page 180): the full components of the SIA manifold are diagramed in fig. 17. The following steps were the protocol applied for the calibration:

I. Potassium permanganate (R) was linked to the selector valve through port 2, Diclofenac sodium (DCS) standard solutions prepared in the range 20 to 250 ppm were linked to the selector valve through ports 3, 4, 5, 6, and 7 while the unknown concentration of DCS which is usually the tablet solution was linked to the selector valve through port 8. Tubings were loaded with their respective reagents by an aspiration run.

II. The syringe pump was filled with acidified water as a carrier (C) by directing the two way valve to the (in-position) mode.

III. 1500 µl acidified water carrier solution were dispensed to wash the holding coil (HC), Reaction coil (RC), the Z photo cell and to adjust the absorbance of the spectrophotometer to zero.

IV. With a 100 µl/s flow rate, 50µl potassium permanganate, and 50 µl of the drug were sequentially aspirated into the holding coil.

V. A short reverse stroke was performed to allow all reagents to mix with a flow rate of 100 µl/s followed by continuous dispensing towards the detector with flow rate of 10 µl/s (D) for 30 seconds and hence the absorbance (A) was recorded.

VI. The steps 2 to 5 were repeated but aspirating the drug sample from the other selector valve ports.

VII. The unknown concentration of the DCS solution was directly recorded from the analysis page in the *FIALab* software.

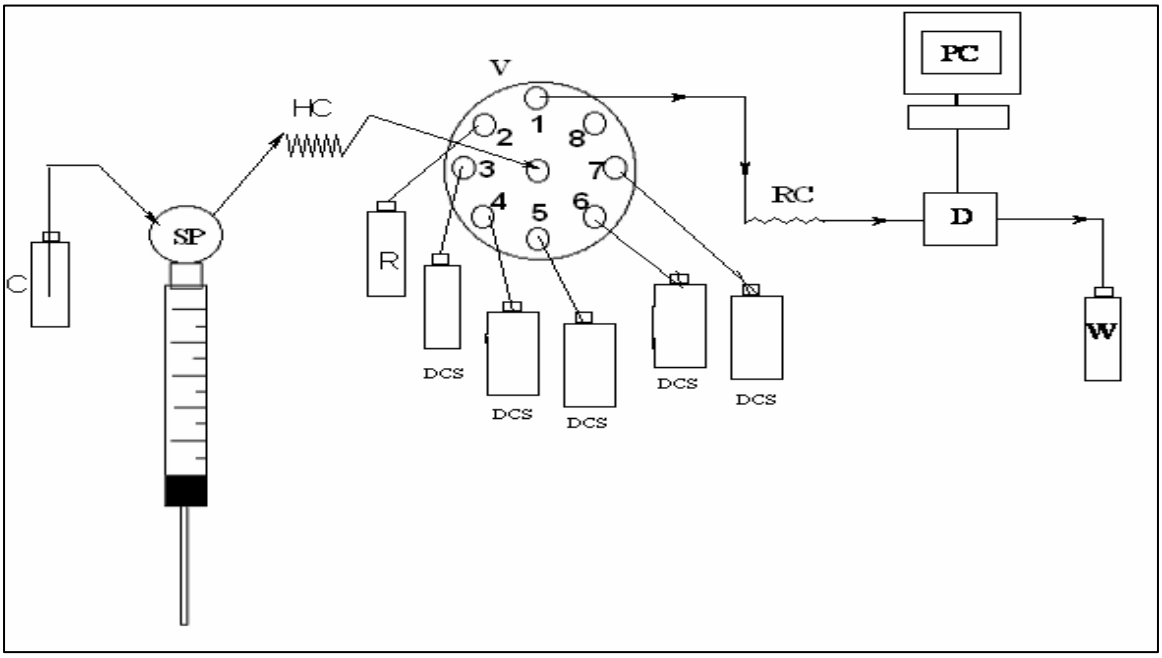


Figure 17: SIA manifold comprised of; A. carrier (water); SP. 5 mL syringe pump; HC. Holding coil; V. eight ports valve; RC. Reaction coil; R. $KMnO_4$; DCS Diclofenac sodium solutions; RC. Reaction coil; D spectrophotometer; PC Computer and W. waste

CHAPTER 3

SEQUENTIAL ININJECTION ANALYSIS SPECTROPHOTOMETRIC METHOD FOR THE ASSAY OF KETOCONAZOLE IN PHARMACEUTICAL PREPARATIONS

3.1 KETOCONAZOLE (KC) LITERATURE REVIEW

Ketoconazole (KTZ), cis-1-acetyl-4-[4-[[2-(2, 4-dichlorophenyl)-2-(1H-imidazole 1ylmethyl)-1,3-dioxolan-4-yl] methoxy] phenyl] piperazine (Scheme 2) is a highly effective broad spectrum antifungal agent. It is used to treat a wide variety of superficial and systemic mycoses and has the advantage over other imidazole` derivatives of producing adequate sustained blood levels following oral administration [48]. KTZ has been evaluated against *Trypanosoma cruzi*, the causative agent of Chagas disease, and it was found to be very effective than other types of drugs [49-52]. Chagas disease an illness which currently afflicts over 17 million people in Latin America and ranks as the third largest parasitic disease worldwide after malaria and schistosomiasis [53]. Recently, new drugs of KTZ that is coordinated to transition metals, such as Ru, Rh, Cu, Au, and Pt, result in a remarkable enhancement of the biological activity as anti *Trypanosoma cruzi* [54, 55]. The characterization of these complexes was achieved through NMR, EPR, IR, UV-vis, elemental analysis, and also an X-ray diffraction study. The biological activities of

these complexes against the epimastigote form of *Trypanosomes cruzi* were also evaluated indicating that all of them inhibit the proliferation of the parasite [56]. Some methods have been reported for its determination including:

Potentiometry: It is the official methods according to British and US pharmacopoeia monographs the method is based on the potentiometeric titration of the drug solution in anhydrous acetic acid with 0.1 M Perchloric acid and detection of the equivalence point potentiometrically [57-58].

Spectrophotometry: Khalil Farhadi and Ramin Maleki proposed a new spectrophotometric method for the determination of ketoconazole in pharmaceutical preparations the method is based on the coupled redox-complexation reactions, which proceed in the ketoconazole-iron (III) and 1, 10-phenanthroline systems and monitoring of the colored complex at 512 nm. [59].

P.Y. Khashaba et al suggested Simple spectrophotometric and spectrofluorimetric methods for the determination of antifungal drugs; (clotrimazole, econazole nitrate, Ketoconazole, miconazole and tolnaftate). Spectrophotometric one depends on the interaction between imidazole antifungal drugs as n-electron donor with the p acceptor 2, 3-dichloro-5, 6-dicyano- 1, 4-benzoquinone (DDQ) in methanol or with *p*-chloranilic acid (*p*-CA) in acetonitrile [60].

Abdel-Gawad F. M, established that ketoconazole reacts with iron (III) chloride in the presence of potassium thiocyanate to form a pink complex (2:1) that is soluble in 1, 2-dichloroethane with a maximum absorbance at 510 nm. [61].

Mirjana P. Vojec, et al studied the acid-base equilibria of a diprotic, slightly hydrosoluble base ketoconazole in homogeneous and heterogeneous water systems. The determinations were performed at 25 ºC at a constant ionic strength of 0.1M (NaCl). The acidity constant K_{a1} was determined by potentiometric (Pk_{a1} 3.20) and spectrophotometric (pK_{a1} 3.26) methods. A pK_{a2} constant of 6.10 was obtained based on the equilibrium constants pK_{s0} 4.84 and pK_{s1} –1.26, determined in a heterogeneous ketoconazole system. The obtained values of the constants served to calculate the solubility and the distribution of the equilibrium forms of ketoconazole as a function of pH. On the basis of the distribution of the equilibrium forms of ketoconazole, a spectrophotometric method for the determination of its content in commercial tablets was developed. The determinations were performed at 225 nm in 0.1 M HCl. The method is simple and rapid and enables the direct spectrophotometric determination of the content of ketoconazole without previous isolation. [62].

F. Jalali & A. Afshoon presented a sensitive spectrofluorimetric method for the detection of ketoconazole, based on formation of a complex between ketoconazole and β-cyclodextrin, the formation of this complex was followed by spectrofluorimety. The inclusion of ketoconazole in β-cyclodextrin cavity enhanced the native fluorescence of the drug. [63].

Erika Rosa Maria Kedor-Hackmann et al developed a spectrophotometric method for quantitative determination of ketoconazole in commercial and simulated emulsion formulations, the method is based on quantitative first-derivative UV spectrophotometric determinations using the zero-crossing method at 257 nm, with methanol as background solvent. [64].

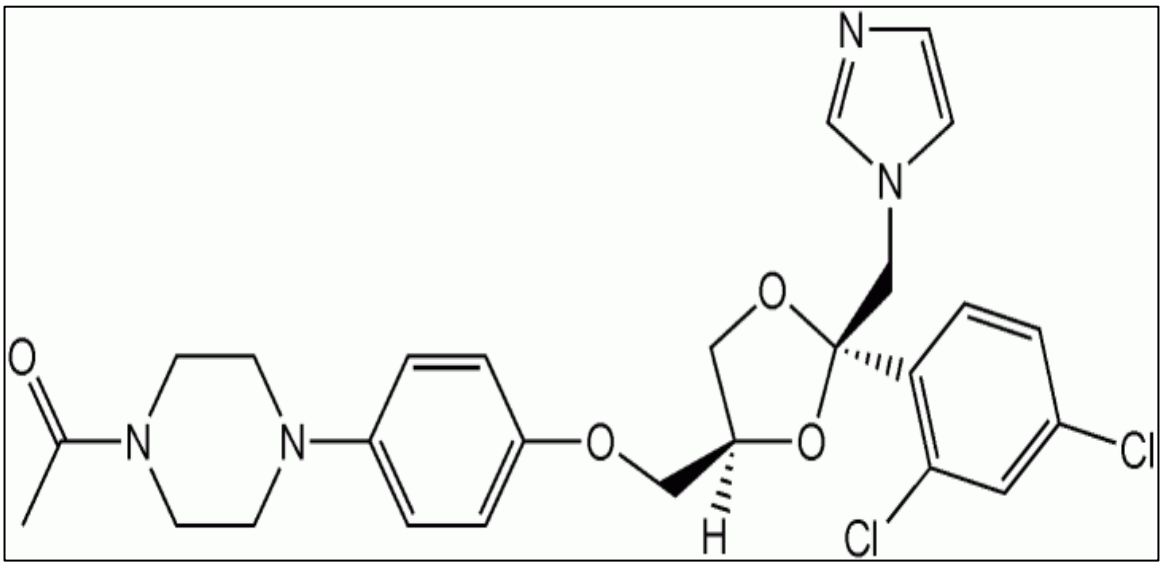
F. Jalali et al described spectrofluorimetric determination of Ketoconazole in Cetyltrimethyl-ammonium Bromide medium; the method was successfully applied to the determination of low concentrations of ketoconazole in pharmaceutical preparations and blood serum sample. [65].

Complexation; F.M. Abou-Attia et al developed a spectrophotometric method for the determination of three pharmaceutical piperazine derivatives, namely ketoconazole (KC), trimetazidine hydrochloride (TMH) and piribedil (PD). This method is based on the formation of yellow orange complexes between iron (III) chloride and the investigated drugs. [66].

El-Ragehy Nairman A. and El-Saharty Yasser S., Investigated ketoconazole copper(II) and cobalt(II) complexes and their spectrophotometric applications, Ketoconazole, as a ligand, reacts quantitatively with copper(II) and cobalt(II) to form blue-colored, stable complexes in dichloromethane. These complexes can be spectrophotometrically measured at 720 and 612.5 nm in the case of Cu (II) or Co (II), respectively. [67]

Chromatography; Adela Arranz et al described Capillary zone electrophoresis (CZE) method for simultaneous determination of a mixture of three imidazolic antifungal drugs. [68]

E.M. Abdel-Moety et al developed High-performance liquid chromatographic technique for the determination or some azolcsantifungals namely, clotrimazole (CZ), ketoconazole (KZ) and fluconazole (FZ), in pure forms and in pharmaceutical formulations. [69]



Scheme 2: Ketoconazole drug

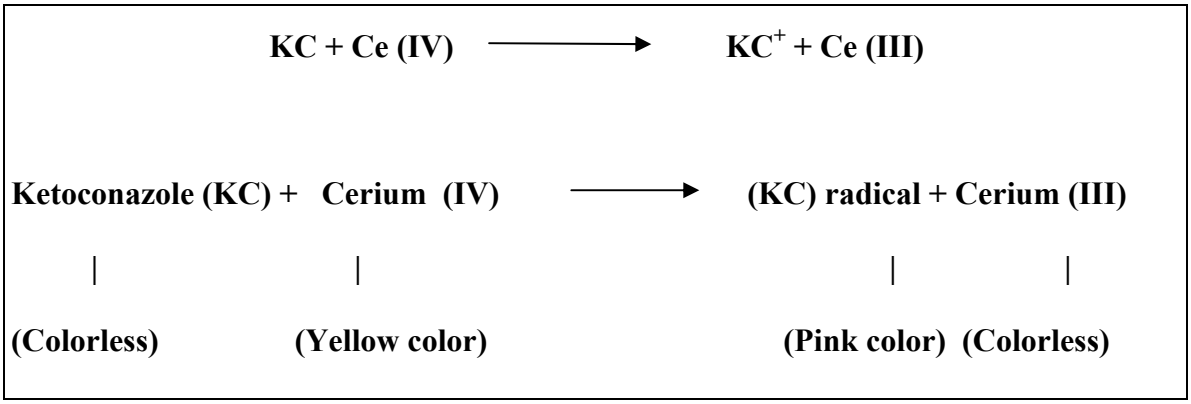
3.2 RESULTS AND DISCUSSION

3.2.1 Reaction Mechanism

The present system is based on the oxidation of the Ketoconazole with cerium (IV) in sulfuric acid media and monitoring the oxidized coloured form of the drug spectrophotometrically utilizing the sequential injection analysis technique. The oxidized form of the drug was found to be stable for more than two minutes and identified as a mono-radical species the absorbs at the wavelength of maximum absorbance at 492 nm.

A recent study on the electro-oxidation of KCZ in aqueous and non-aqueous media proved that the KCZ is initially oxidized reversibly with the loss of one electron to the cation radical ($KC^{\cdot+)}$. This radical can be stabilized by resonance, resulting in the observed pink-red color product. The stability of the cation radical is completely dependent on the conditions of solution, so that it gradually decays via a chemical reaction in polar solvent and/or in weak acidic aqueous media. Also, it was found that, $KC^{\cdot+}$ can be further oxidized with the loss of the second electron to give some stable products [59].

On basis of the results mentioned above, the chemical oxidation of KCZ was investigated with cerium(IV) as an oxidant in sulfuric acid media. The newly developed spectrophotometric method utilizes the SIA as the major technique for solution handling graphically represented as in Scheme 3



Scheme 3: Reaction scheme for the proposed SIA method for the determination of Ketoconazole

3.2.2 Method optimization

A preliminary investigation was conducted to find out the experimentally possible range of the levels of variables presumed to be potentially affecting on the efficiency of the SIA method, namely flow rate, sulfuric acid concentration and cerium (IV) concentration. Three chemometrics approach were applied for the purpose of SIA system optimization, namely univariate, factorial design and multisimplex.

3.2.2.1 Uni-variate Method Approach

The method for the determination of Ketoconazole by Sequential injection was optimized using the uni-variate method approach The univariant, one-variable-at-a-time, method optimizes one variable using different levels of that variable and fixed levels of other variables in a system [70]. In this concern, a number of experiments were conducted using sulphuric acid (0.35, 0.4, 0.45, 0.5, 0.55, and 0.6 mol/l), cerium (IV) (50, 100, 150, 200, 300, 450, and 600 ppm), and different flow rates ranging from 10 to 50 µl/s.

3.2.2.1.1 Delay time

Delay time ensures more intensely colored products formation; this subsequently enhances the sensitivity of the method but on the other hand the colored products may not be so stable and decomposition of the colored product may take place before monitoring of the absorbance and this will affect the sensitivity of the method. In case of Ketoconazole the delay time was varied between 30 to 180 seconds and it was found that the pink

Ketoconazole radical is stable up to 120 second without any significant decrease in the absorbance. (Table 4 and figure 18)

Table 4 The effect of delay time on the absorbance of Ketoconazole radical

EXP. NO.	DELAY TIME (S)	ABSORBANCE*
1	30	0.333
2	90	0.338
3	120	0.327
4	150	0.298

*Average of three determinations

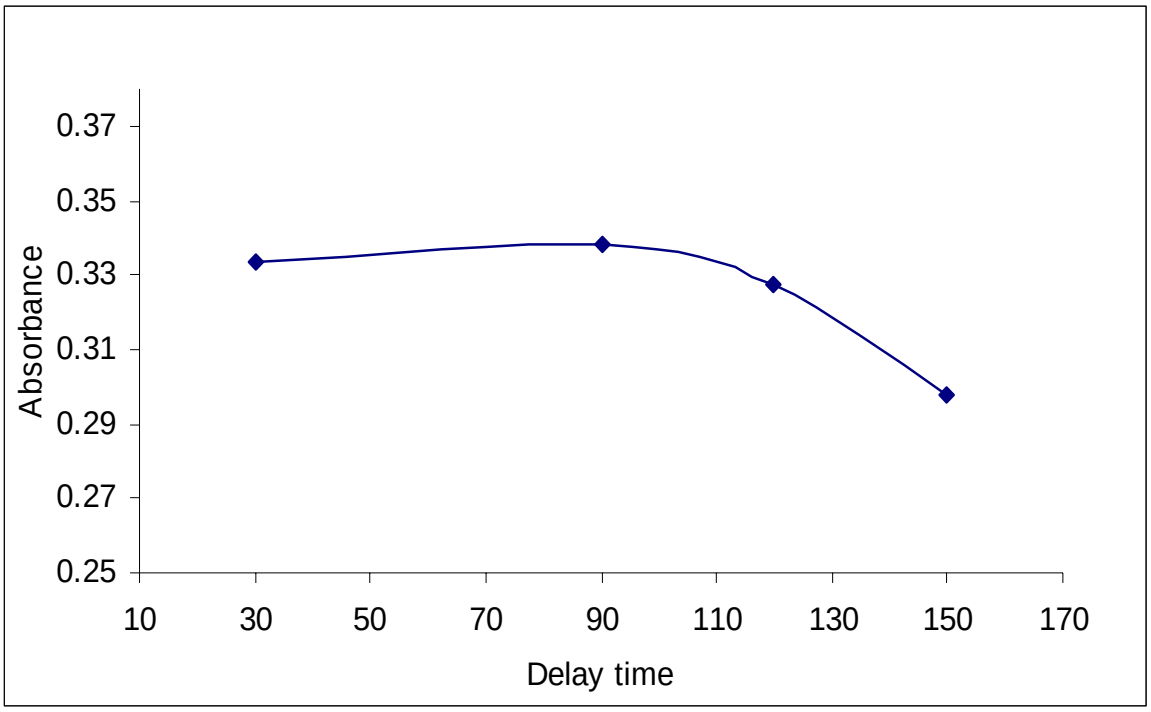


Figure 18 The effect of the delay time on the absorbance peak height of 50 ppm KC

3.2.2.1.2 Flow rate

The effect of the flow rate on the peak absorbance was investigated and found to be a major affecting parameter on the absorbance. Low flow rate ensures that the colored products spend more time in the flow cell and absorbance is well measured, it also provides more time for the colored complex to be formed in route to the detector. The optimum flow rate for the proposed SIA method was observed to be 30 µL/s. There was a drop in absorbance at higher flow rate because of the high speed of the colored KCZ radical produced through the flow cell and hence very low sensitivity (table5 and figure19).

Table 5 The effect of flow rate on the absorbance of Ketoconazole radical

EXP. NO.	FLOW RATE ML/S	ABSORBANCE*
1	10	0.221
2	20	0.291
3	30	0.456
4	40	0.338
5	50	0.159

*Average of three determinations

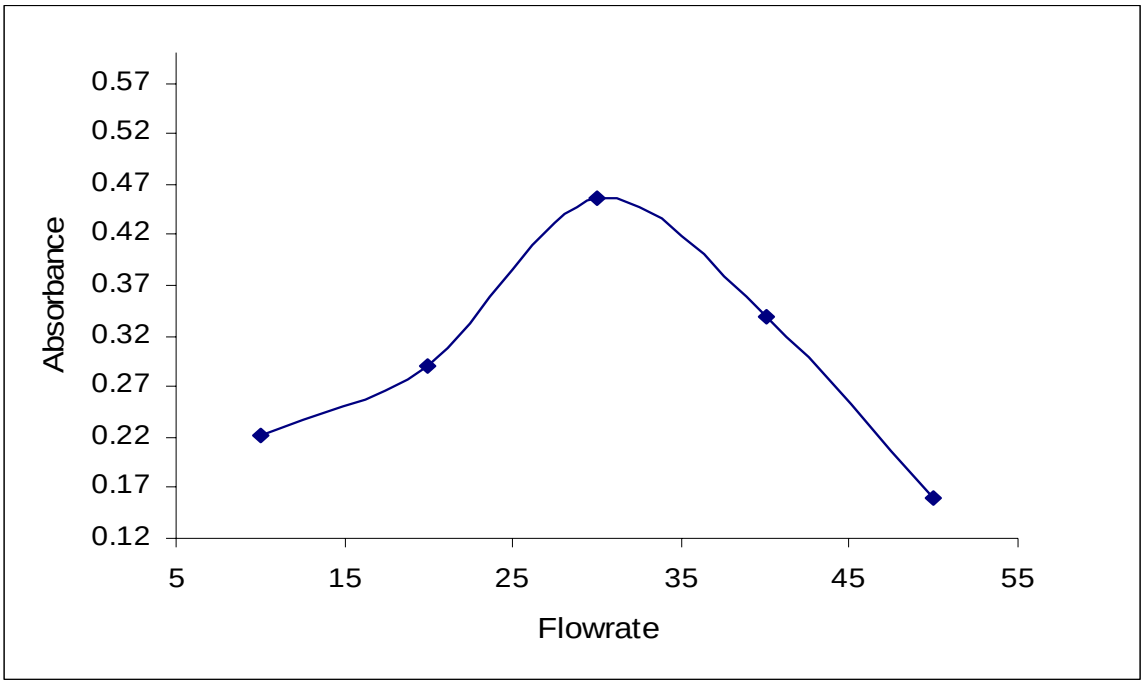


Figure 19 The effect of the flow rate on the absorbance peak height of 50 ppm KC

3.2.2.1.3 Sulfuric acid concentration

The concentration of sulfuric acid is one of the significant parameters affecting the absorbance of the product, since the oxidation power of Ce (IV) depends on the acidity of the medium. Long series of sulfuric acid concentrations were prepared for the purpose of studying the effect of the acid concentration on KC radical absorbance. The optimum acid concentration was found to be 0.55mol/l (table 6 and figure 20)

Table 6 Effect of the acid concentration on the absorbance of KC radical

EXP. NO.	ACID M	ABSORBANCE*
1	0.35	0.313
2	0.40	0.315
3	0.45	0.314
4	0.50	0.338
5	0.55	0.350
6	0.60	0.304

*Average of three determinations

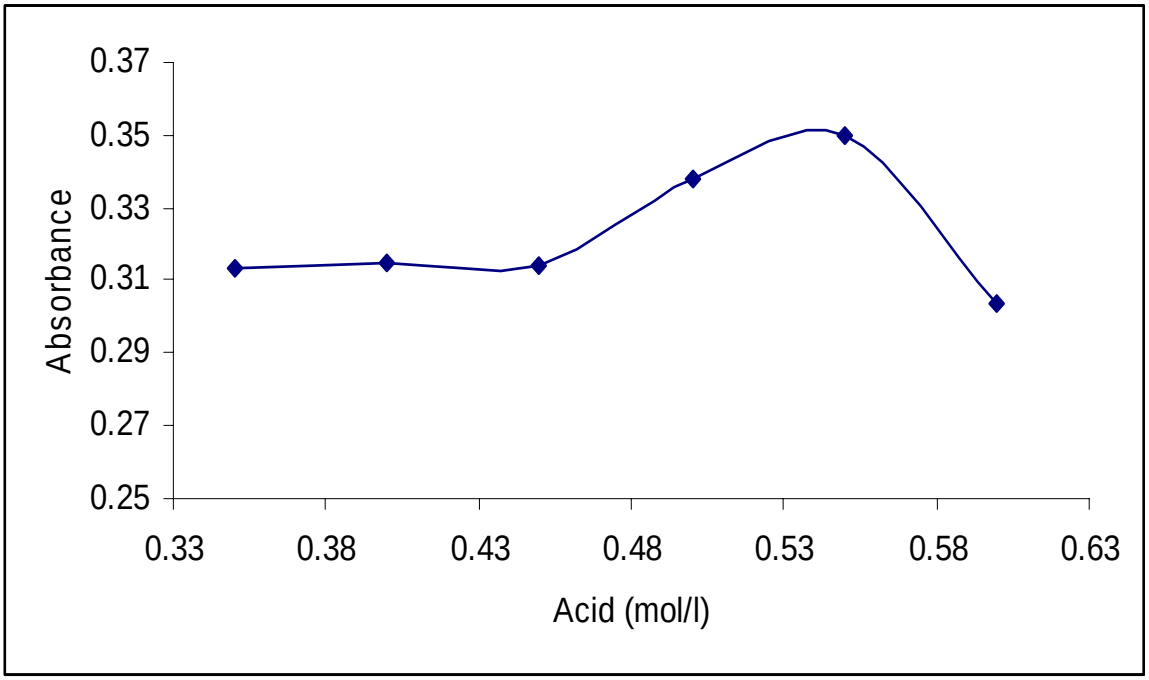


Figure 20 The effect of the acid on the absorbance peak height of 50 ppm KC

3.2.2.1.4 Cerium (IV) concentration

Cerium (IV) is a powerful and strong oxidizing agent. It is important to optimize the concentration of cerium(IV) in case of Ketoconazole, it was found that the color of the chemically produced cation radical immediately disappeared in the presence of excess Ce(IV), probably due to further oxidation of KC^{+} to colorless dication (KC^{2+}) (table7 and fig. 21.[59]

Table 7 The effect of the Ce (IV) concentration on the absorbance of KC radical

EXP. NO.	CERIUM PPM	ABSORBANCE*
1	50	0.276
2	100	0.331
3	150	0.352
4	300	0.350
5	450	0.270
6	600	0.242

*Average of three determinations

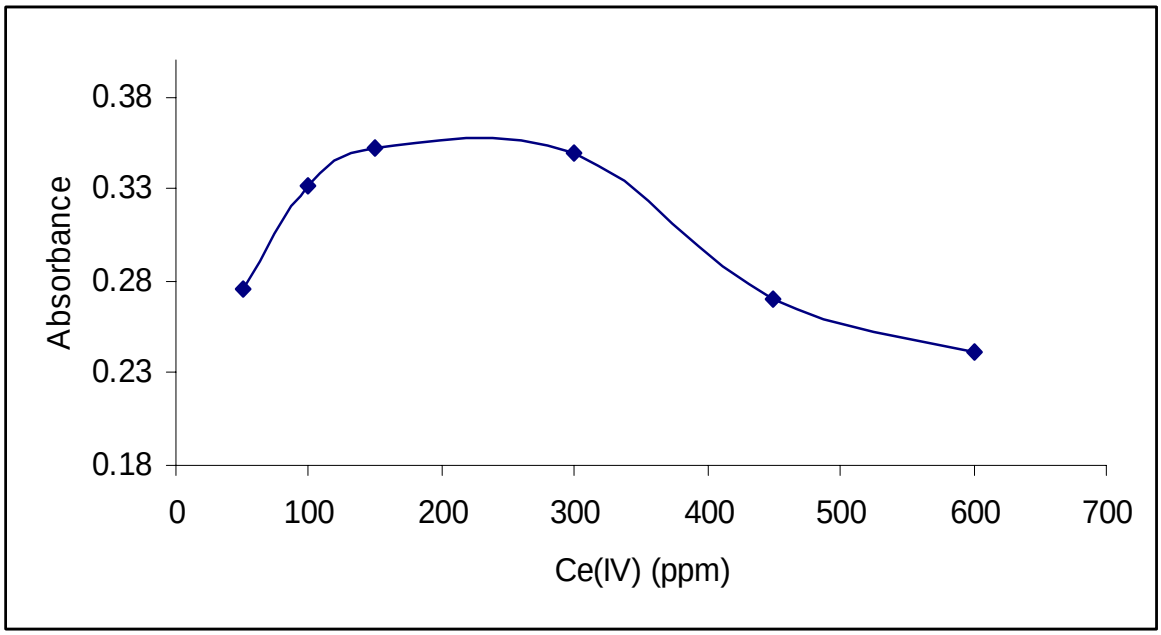


Figure 21 the effect of Ce (IV) concentration on the absorbance peak height of 50 ppm KC

3.2.2.2 Factorial Design

In this work, three level factorial designs were applied to the three variables. The highest, encoded as (+1), and the lowest, encoded as (−1), levels of the factorial design were obtained from the preliminary investigation using univariant approach, while the medium level, encoded as (0), was mathematically calculated. A total of twenty-seven experiments, as a result of 3^3 full factorial designs, were conducted. Table 8 presents the adopted matrix and the experimental results. By the means of SigmaPlot® software, the results obtained for the response versus each two variables were interpolated and graphed (Figs. 22, 23 and 24). Fig. 22 reveals that the response significantly increases as cerium (IV) concentration and flow rate increases this indicating that cerium (IV) concentration and flow rate significantly affects the response and the maximum absorbance is obtained at the medium values of Ce (IV) as well as flow rate, and this point is clear explained with the contour plot Fig. 25. Fig. 23, shows that at the two ends of the Ce(IV) concentration the response is decreased to its lowest values and this observation can be explained as follow: at the lower value there were no sufficient amount of Ce(IV) and not all of the drug amount was oxidized and at high of Ce(IV) value the further oxidation of KC^{+} to the colorless KC^{++} takes place. The highest response is obtained at medium values of Ce(IV) and the acid. Fig. 24 shows that the effect of acid concentration on the response is lower than the effect of flow rate and the response is significantly increases with the flow rate and reaches the highest at the medium value of the flow rate.

Figure 25 contour plots of the data (cerium and flow rate) where the contour different colored zones show the response different values. The pink zone indicates the largest

response value and it will be achieved if the levels of cerium(IV) and flow rate are both set at medium levels. There is no curve in the contour lines indicating the absence of the interaction between cerium and flow rate. Fig 26 and 27 the presence of non linear contour lines shows strong interaction between cerium and the acid (fig26) and also between the acid and flow rate (fig 27).

Table 8 A 3^3 factorial design matrix with experimental results (responses)

Experiment No.	Factors			*Response
	[CeIV]	[Acid]	F.r.μ / s	
1	-1	-1	-1	0.150
2	-1	-1	0	0.212
3	-1	-1	+1	0.187
4	-1	0	-1	0.164
5	-1	0	0	0.260
6	-1	0	+1	0.230
7	-1	+1	-1	0.186
8	-1	+1	0	0.257
9	-1	+1	+1	0.133
10	0	-1	-1	0.188
11	0	-1	0	0.300
12	0	-1	+1	0.289
13	0	0	-1	0.183
14	0	0	0	0.288
15	0	0	+1	0.233
16	0	+1	-1	0.197
17	0	+1	0	0.340
18	0	+1	+1	0.239
19	+1	-1	-1	0.071
20	+1	-1	0	0.169
21	+1	-1	+1	0.166
22	+1	0	-1	0.078
23	+1	0	0	0.213
24	+1	0	+1	0.233
25	+1	+1	-1	0.096
26	+1	+1	0	0.246
27	+1	+1	+1	0.248

*Average of three determinations

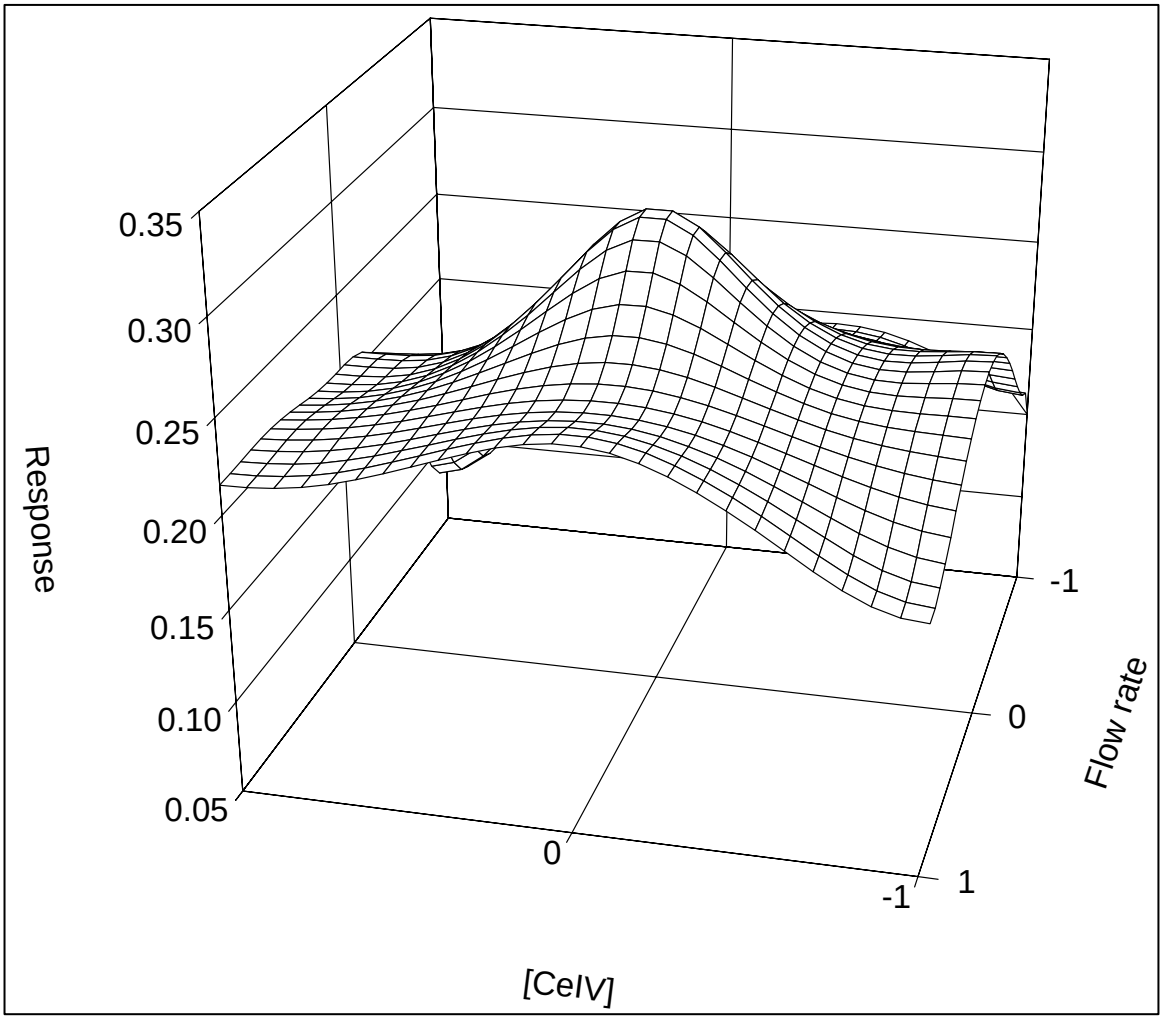


Figure 22 Surface plot of the response versus Ce (IV) concentration (ppm) and flow rate

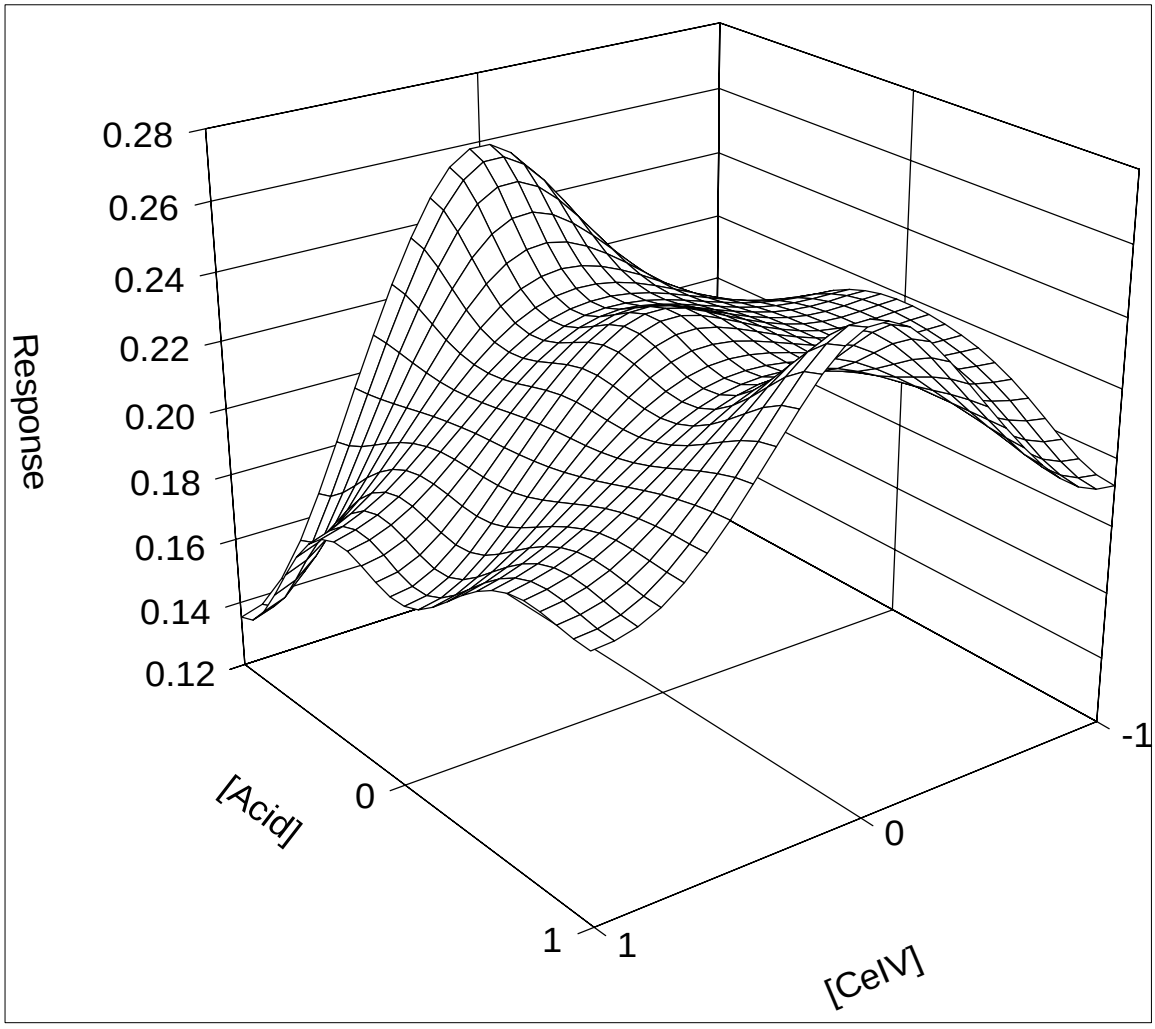


Figure 23 Surface plot of the response versus sulphuric acid concentration (mol/l) And Ce(IV) concentration (ppm)

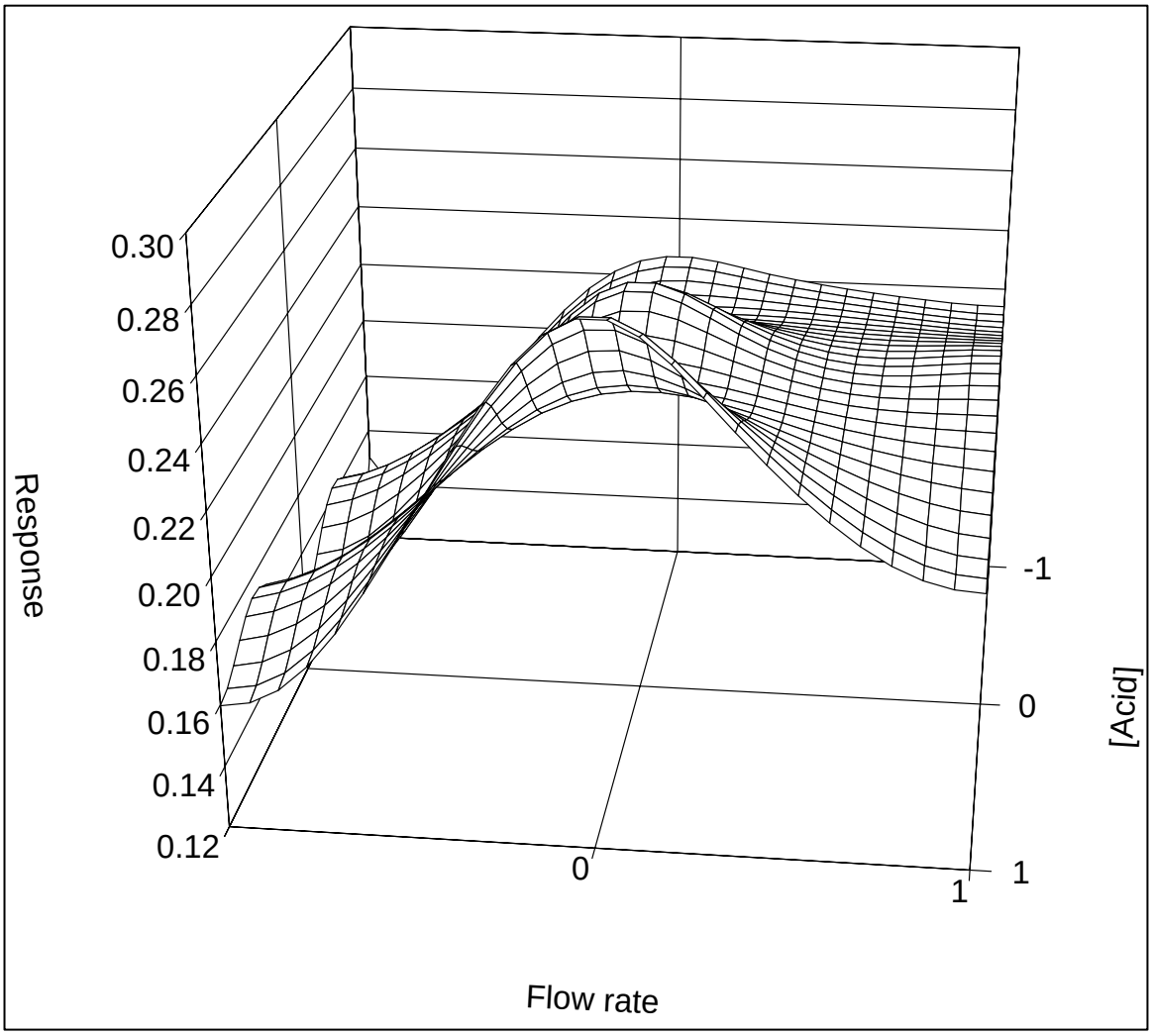


Figure 24 Surface plot of the response versus sulphuric acid concentration (mol/l) and flow rate µl/s

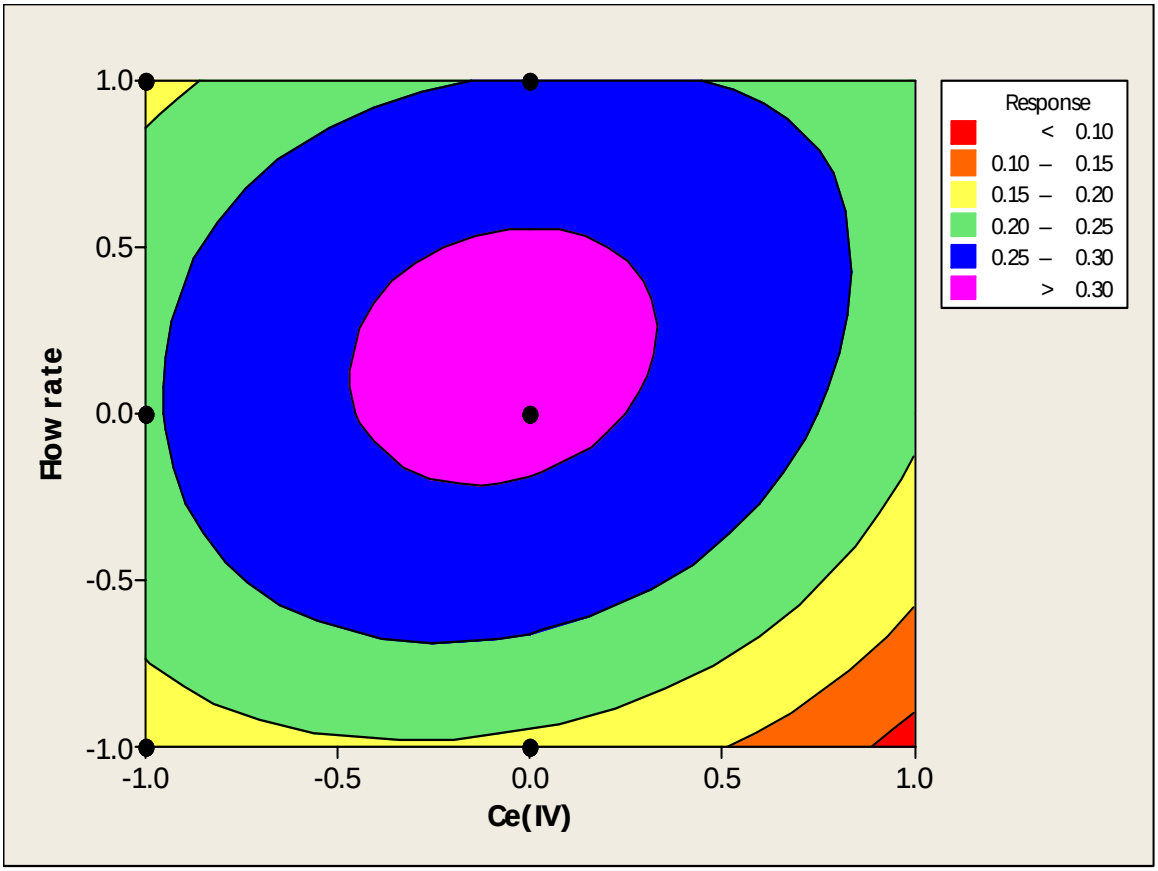


Figure 25 Contour Plot of Response vs. Flow rate, Ce(IV)

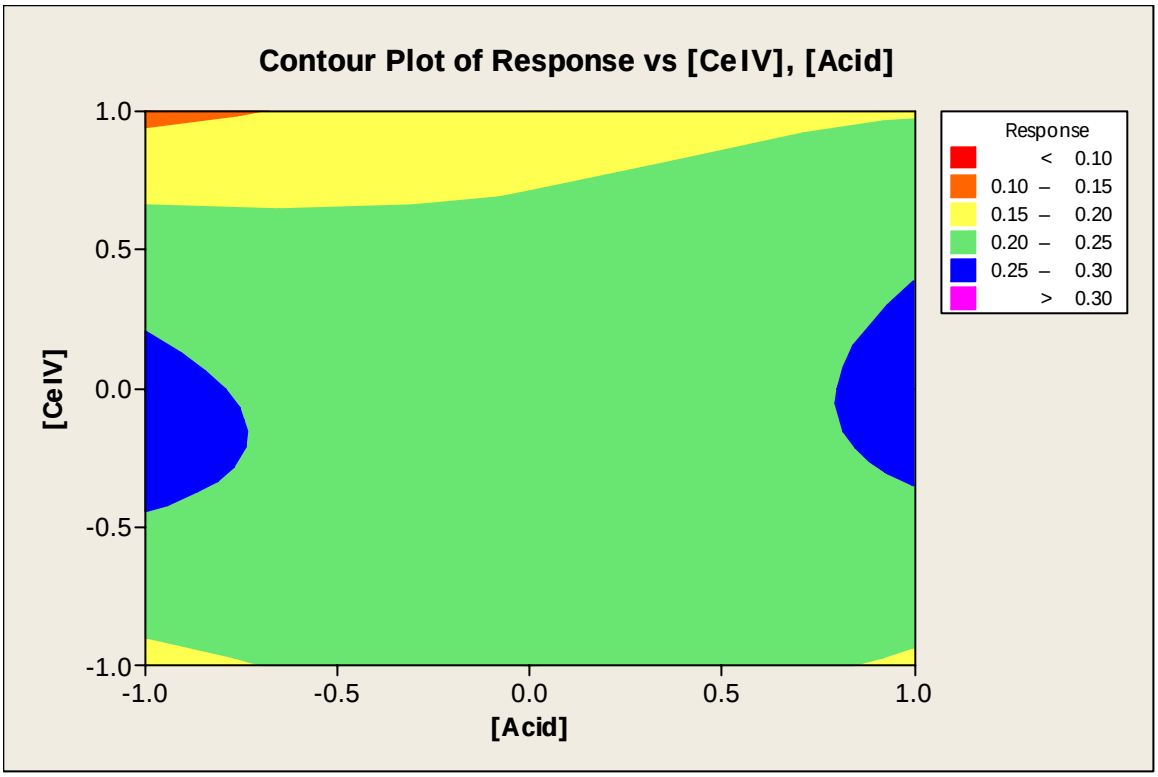


Figure 26 Contour Plots of Response vs. Ce(IV), acid

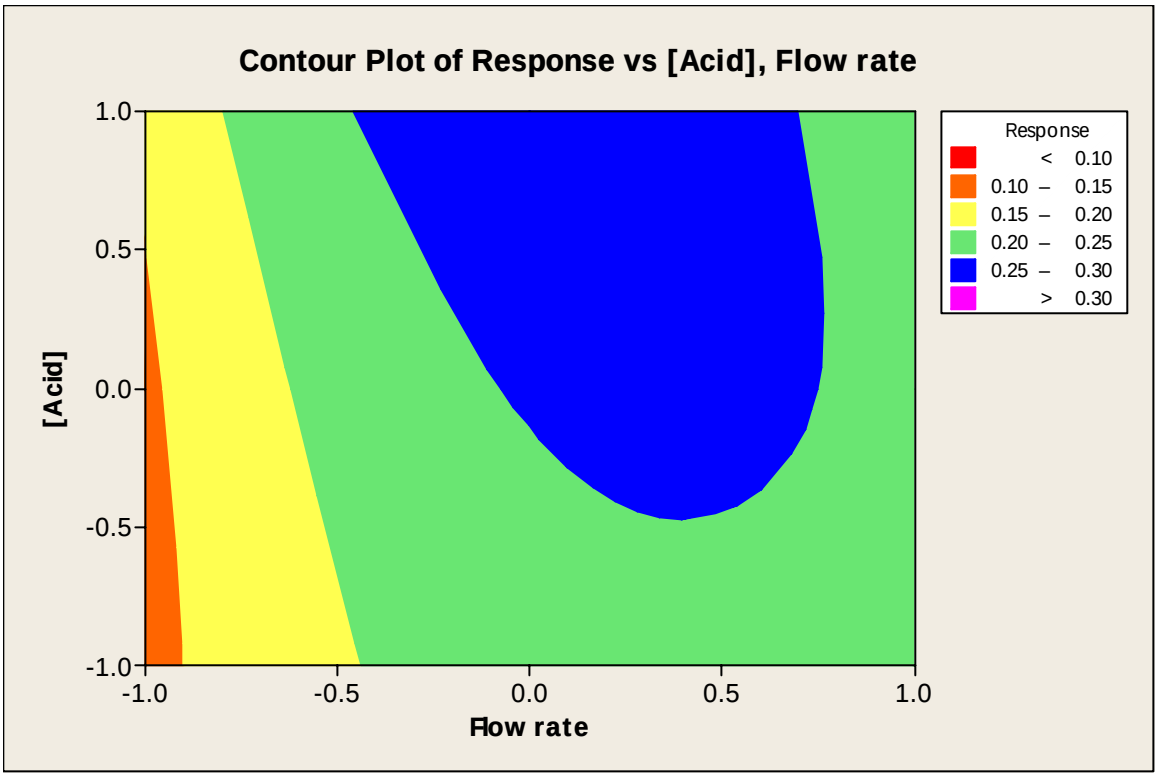


Figure 27 Contour Plots of Response vs. Acid, flow rate

The ANOVA principle provides the basis of the statistical analysis aspect of factorial design by providing the mathematics for test statistic construction and derivation based on response model specification and the underpinning statistical theory [71].

Results obtained by using Minitab, Table 9 shows the ANOVA table produced by Minitab software. The p values for Cerium(IV) concentration, sulfuric acid concentration, and flow rate are zero. That means all factors are significant. In other words, cerium(IV), sulfuric acid, and flow rate are significantly affecting the response. Also the p values for two factor interaction and three factor interaction are smaller than 0.05 (α). That means the interaction have a significant effect on the response.

The interaction between the factors can be visualized with an interaction plot, Figures (28, 29, and 30). The interaction plot graphs the means of the replicates, organized based on the three levels of the factors. The presence of non-parallel lines in the interaction plot is indicative of the occurrence of an interaction effect and that the interaction is reflecting a non-uniform order of response. [71].

Table 9: ANOVA table by using Minitab

Analysis of Variance for Response

Source	DF	SS	MS	F	P
[CeIV]	2	0.230650	0.115325	73.22	0.000
[Acid]	2	0.076386	0.038193	24.25	0.000
Flowrate	2	0.688518	0.344259	218.56	0.000
[CeIV]*[Acid]	4	0.048150	0.012037	7.64	0.000
[CeIV]*Flowrate	4	0.044475	0.011119	7.06	0.001
[Acid]*Flowrate	4	0.039075	0.009769	6.20	0.001
[CeIV]*[Acid]*Flowrate	8	0.044334	0.005542	3.52	0.007
Error	27	0.042528	0.001575		
Total	53	1.214116			

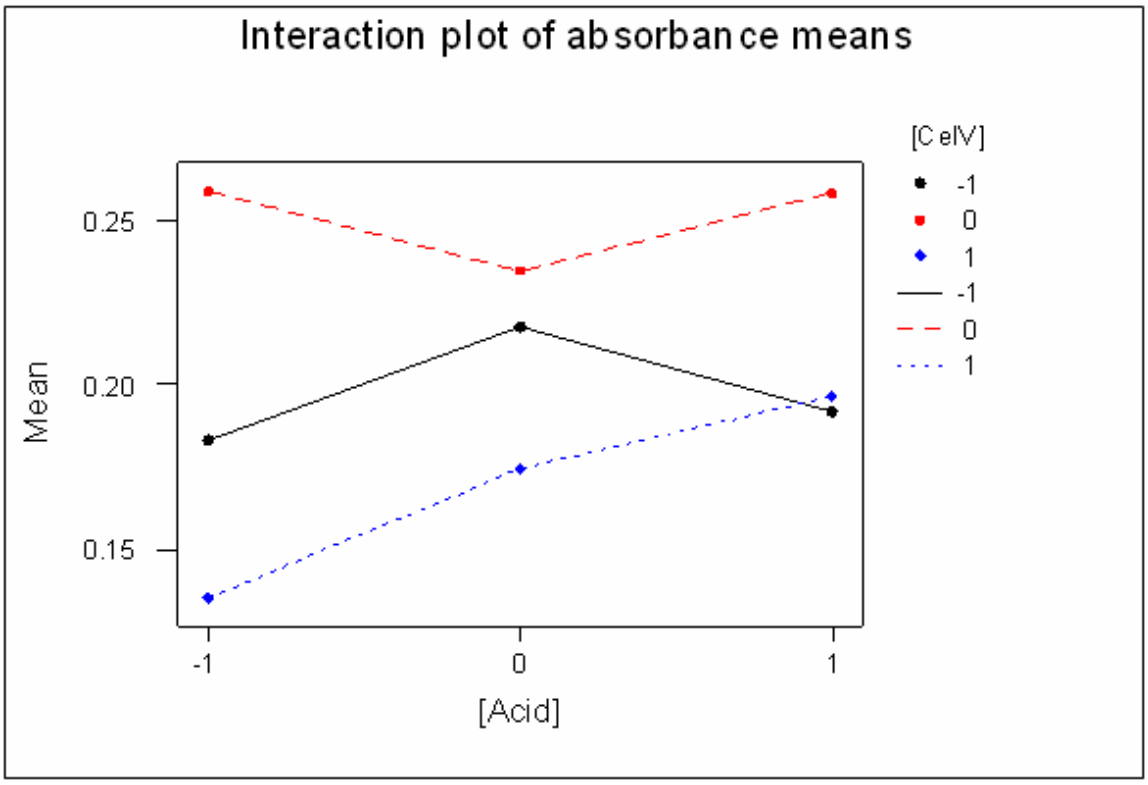


Figure 28: Interaction plot for cerium and the acid showing that the two factors influence each other (non- parallel lines).

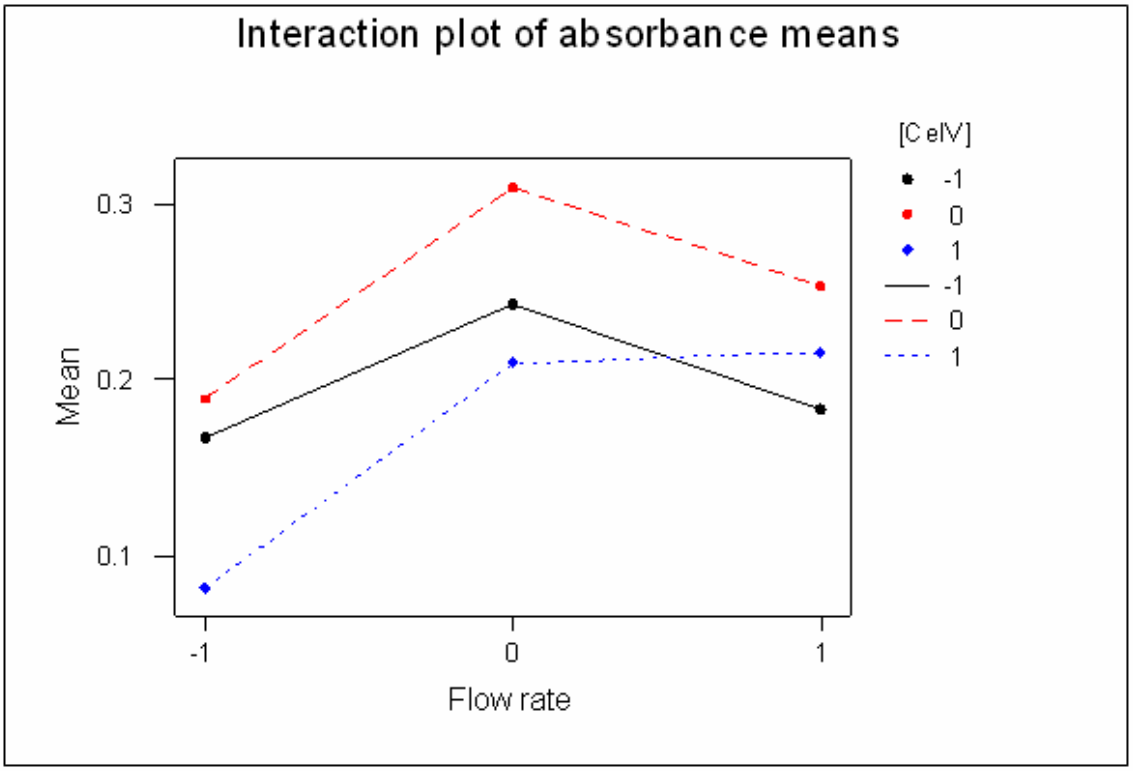


Figure 29: Interaction plot for cerium and the flow rate showing that the two factors influence each other (non- parallel lines)

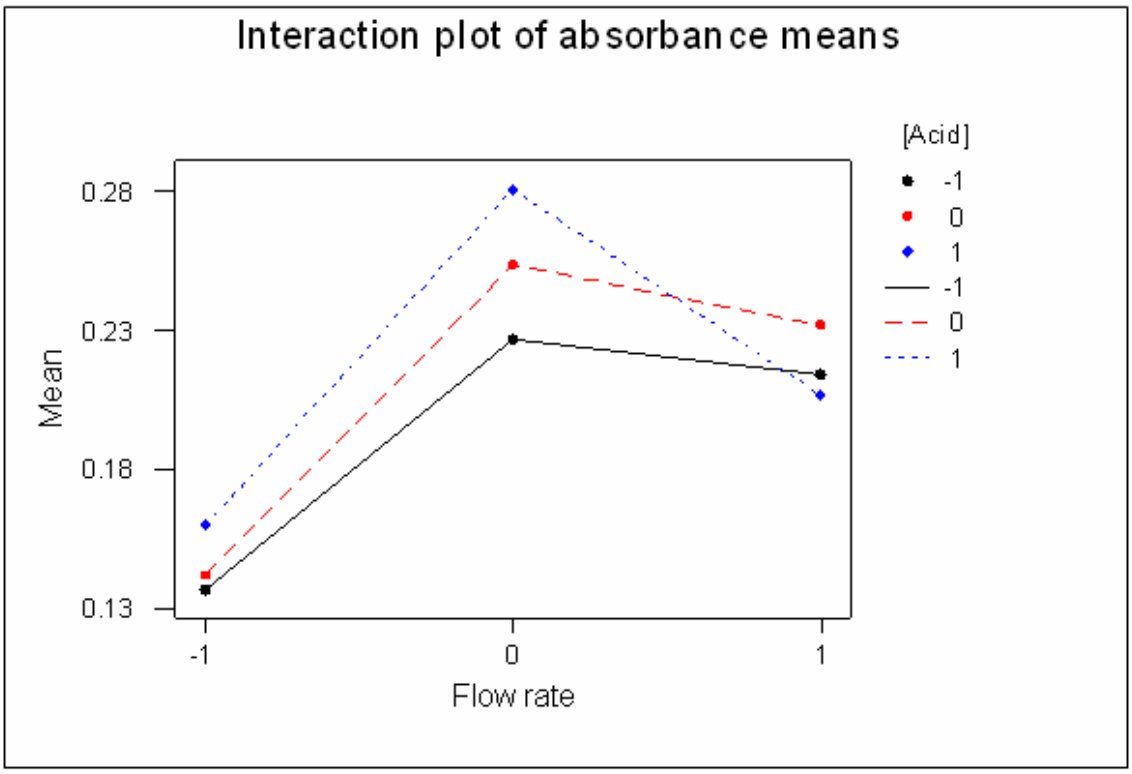


Figure 30: Interaction plot for acid and the flow rate showing that the two factors influence each other (non- parallel lines).

Another ANOVA approach was applied utilizing Minitab software to investigate the main factor effect i.e. the effect factor (Ef) on the response. It is calculated using equation 1. Therefore, a 2^3 factorial design was adopted to calculate the effect factors as in table 10. The interaction effect factor explains the level of the interaction effect between variables on the response of a system. The main and interaction effect factors were calculated and the results obtained are introduced in table 11 [71]. The main effect factors disclose that the cerium(IV) slightly and negatively affects the response while acid concentration and flow rate significantly and positively affect the response. The two-variable interaction effect factor between acid concentration and flow rate is higher than that between flow rate and the cerium(IV) concentration or acid concentration and Ce(IV) concentration and so for the three variable interactions.

Effect of each factor = (*effect contrast*) / (2^{k-1}) (1)

Whereas (*effect contrast) can* be calculated using table 12, (k) is number of factors

Table 10: 2^3 factorial design matrix

Combination	Ce(IV)	Acid	Flow rate	*Response
(1)	-	-	-	0.150
a	+	-	-	0.071
b	-	+	-	0.186
ab	+	+	-	0.096
c	-	-	+	0.187
ac	+	-	+	0.166
bc	-	+	+	0.133
abc	+	+	+	0.248

*Response denotes a triplicate response measurement

Table 11 The effect of variables on response

Effect	**Variable**	**Value**
Main	Ce(IV)	-0.019
	Acid	0.022
	Flow rate	0.058
Tow-variable interaction	Ce(IV)* Acid	0.031
	Ce(IV)* Flow rate	0.066
	Acid* Flow rate	-0.008
Three-variable interaction	Ce(IV)* Acid* Flow rate	0.0037

Table 12 Contrast for a three factor two-level experiment

Effects	Contrast
A	a + ab + ac + abc – (1) – b – c – bc
B	b + ab + bc + abc – (1) – a – c – ac
AB	(1) + ab + c + abc – a – b – ac – bc
C	c + ac + bc + abc – (1) – a – b – ab
AC	(1) + b + ac +abc – a – ab – c – bc
BC	(1) + a + bc + abc – b – ab – c – ac
ABC	a + b + c + abc – (1) – ab – ac – bc

A, B and C denotes the factors

3.2.2.3 MultiSimplex method

The Multisimplex® software package was employed to find out the optimum levels of variables potentially interact, i.e. the acid concentration cerium(IV) concentration, and flow rate. Reference values and steps as the initial inputs of the simplex method are introduced in Table 13. Although Multisimplex® is not restricted by boundaries, some levels are impossible to be conducted practically. Hence, the same ranges of levels of parameters obtained from the preliminary investigation were considered in the Multisimplex®. Up to 20 sequential experiments were practically carried out (table 14) and the response function progress is plotted in Fig. 31. The advantage of the Multisimplex® method over the other simplex methods, i.e. basic simplex, modified simplex and super modified simplex, is that the optimum levels are obtained in less number of trials [72]. The first four trials are proposed based on pure mathematical calculation while the other consequent trials are proposed based on both mathematical calculations and the inputs of the response of a previous experiment. As depicted in Fig. 21, the response starts with low values and sharply increases at experiment 4 and obtains the maximum at experiment 17, it can be concluded that 0.44 mol/l sulphuric acid, 4.4×10^{-4} mol/l cerium (IV) and flow rate 16.202 µl/s are the optimum experimental conditions of the adopted SIA method.

Table 13 Initial inputs of the Multisimplex® (concentration in mol L^{-1})

MultiSimplex inputs	**Acid**	**Ce(IV)**	**Flow rate µl/s**
Reference value	*0.3*	4.8×10^{-4}	*15*
Step	*0.03*	4×10^{-5}	*2*

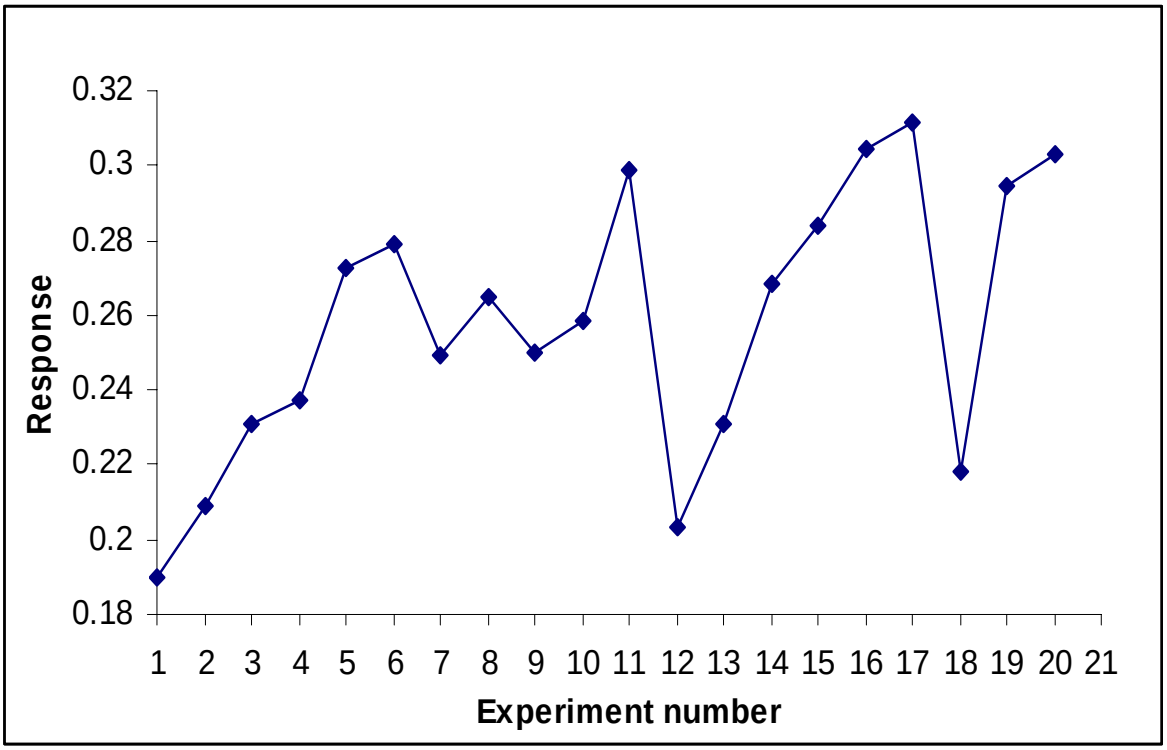


Figure 31 Response function progress of the Multisimplex® optimization

Table 7 Concentration values (mmol in 50 mL solution), flow rate and corresponding absorbance

Trial number	Acid (mmol)	Ce(IV) (mmol)	Flow rate (µl/s)	*Absorbance
1	14.25	0.025	14	0.189741
2	15.75	0.023	14	0.208673
3	14.25	0.023	16	0.230702
4	15.75	0.025	16	0.237138
5	16.25	0.022	16.667	0.2724501
6	17.25	0.021	18	0.2791007
7	15.75	0.023	19.333	0.2494605
8	18.25	0.023	19.555	0.2649777
9	18.417	0.02	21.925	0.2498381
10	20.195	0.02	20.32	0.25874
11	18.713	0.023	16.658	0.2990745
12	18.861	0.024	14.025	0.202994
13	15.947	0.025	15.822	0.2310246
**6RE1	17.25	0.021	18	0.2511369
14	19.133	0.021	19.196	0.2685093
15	18.481	0.02	16.348	0.2839883
16	20.301	0.022	16.801	0.3041627
17	21.827	0.022	16.202	0.3113217
18	20.214	0.022	13.609	0.217985
19	19.403	0.021	17.799	0.2944458
20	21.481	0.024	17.425	0.3027305

*Absorbance reading an average of 3 determinations

**6RE1= Experiment 6 was reevaluated

3.2.3 Method validation

3.2.3.1 Linearity & range

A long series of standard solutions of Ketoconazole were subjected to the optimized SIA method for the purpose of calibration. Beer's law was found to be obeyed in the concentration range of 30–180 μg/mL with weighed regression Abs. (a.u.) = 0.05286 + 0.002687 KC μg/mL the correlation coefficient was 0.9965 indicating good linearity. Figure 32 and 33 shows a typical SIA calibration obtained under the optimum conditions for five consequent injection of five standard solutions of Ketoconazole (30, 60, 90, 120, 150, and 180 μg/mL). Fig. 34 shows the SIAgram obtained under optimal conditions for five standard solutions of Ketoconazole pure samples followed with unknown solution of the same drug in tablet formulation.

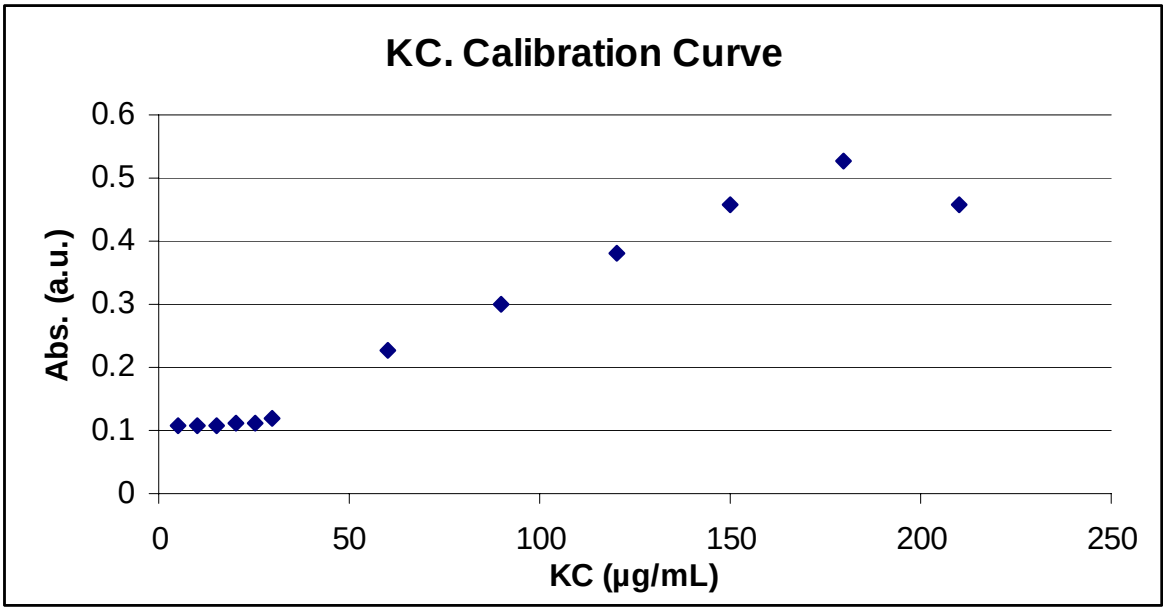


Figure 32: The Absorbance of Ketoconazole radical versus drug concentration calibration plot

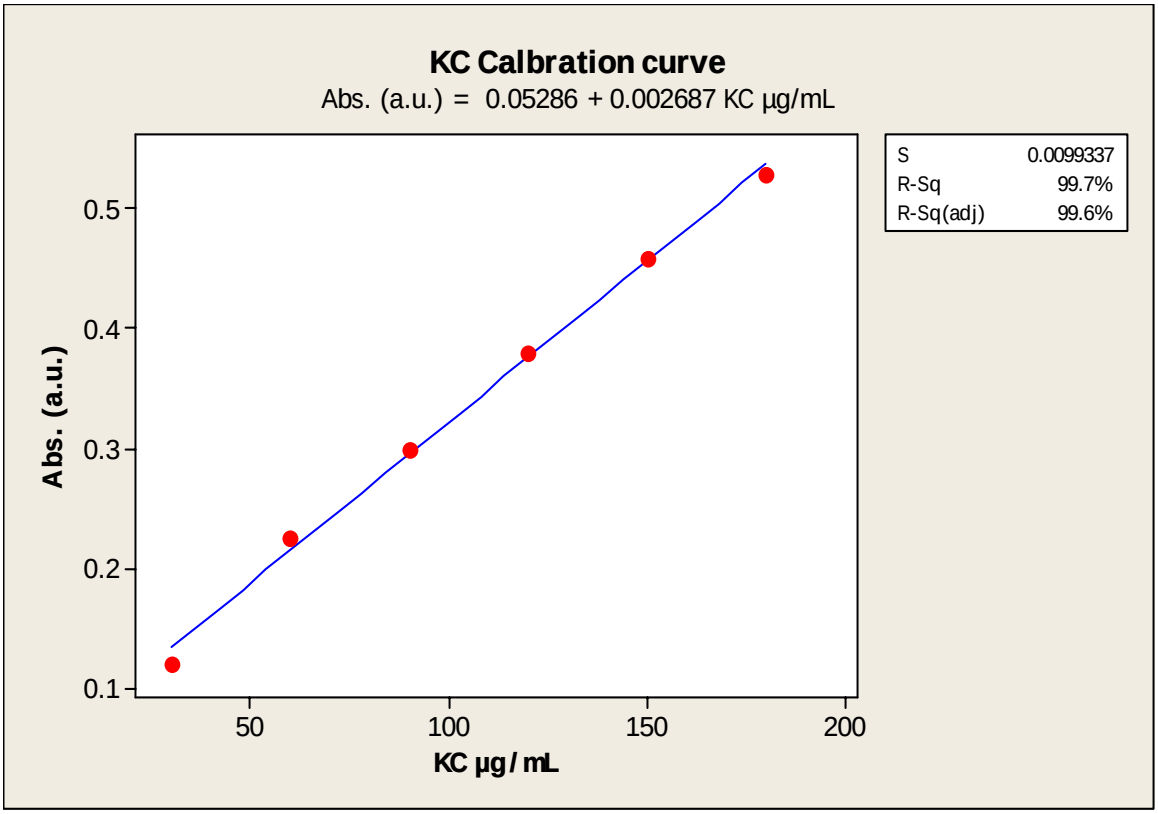


Figure 33: The Absorbance of oxidized form of the drug versus Ketoconazole Concentration fitting plot

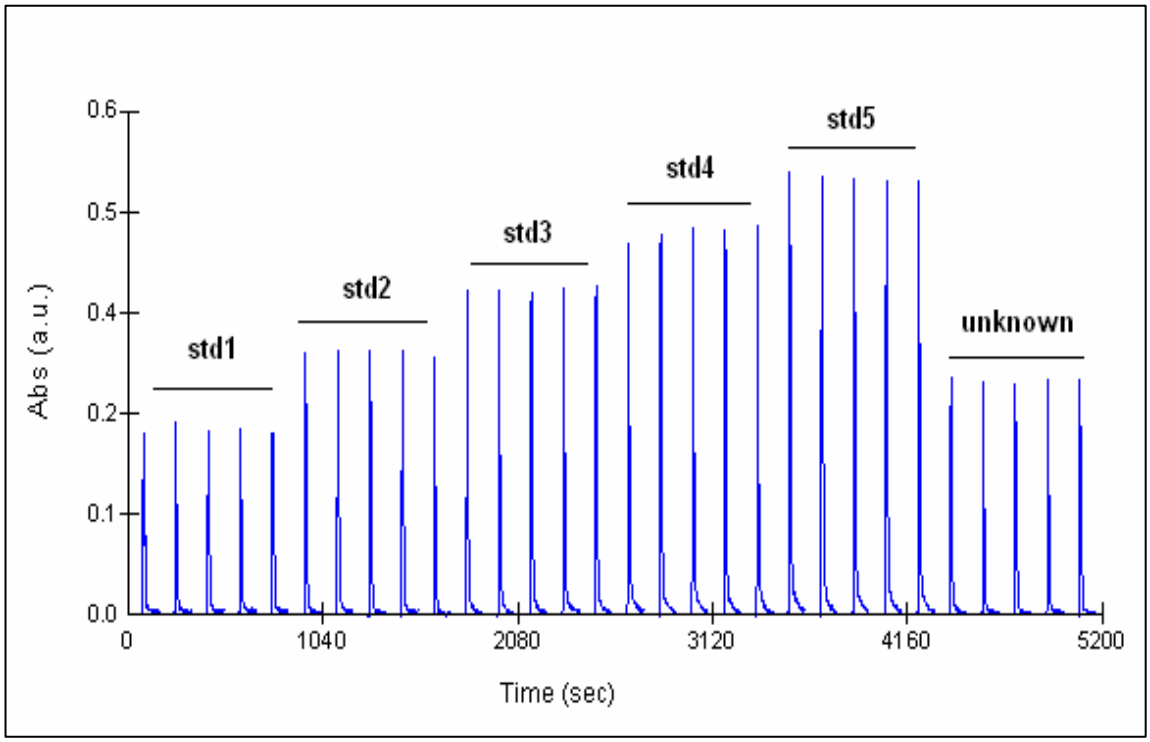


Figure 34 The SIA output obtained with proposed system, providing a Ketoconazole calibration plot between 30–180 μg/ml (std = standard), the peak for tablet unknown solution is shown.

3.2.3.2 The accuracy

The accuracy was examined by analyzing tablets formulations. The obtained results were realized by the US Pharmacopoeia (USP) method. USP provided a classical potentiometric titration method by Perchloric acid for Ketoconazole assay in tablets formulation. Analysis for the tablet sample was repeated three times, and the relative standard deviation (RSD) was calculated. The t-test values were also calculated. The results obtained are introduced in table 15. The obtained results indicating that the provided SIA method is accurate and repeatable as the t-test calculated were always less than the tabulated values at 95% confidence level.

Table 15 Results obtained by the SIA and US Pharmacopoeia methods for the analysis of Ketoconazole in tablets sample

Drug	Supplier	Contents (mg)	Samples	Mean recovery ±RSD (%)[a]		t[b]-test value
				SIA method	USP[c] method	
Nizoral	Janssen Pharmaceutica N. V., Turnhoutseweg 30, B-2340 Beerse, Belgium	Ketoconazole 200 mg per tablet	Ketoconazole 40 mg/l	99 ± 1.6	99.8 ± 1.3	1.1 (t Critical = 2.13)
			Ketoconazole 80 mg/l	99.8 ± 1.6	99.5 ± 1.3	0.6 (t Critical = 2.13)
			Ketoconazole120 mg/l	99.8 ± 1.4	100 ± 1.0	0.2 (t Critical = 2.13)

[a] Relative standard deviation for 5 replicates.

[b] Student t-test values.

[c] United State Pharmacopoeia.

3.2.3.3 The intermediate precision

The intermediate precision of the SIA method was examined by analyzing the same solutions 5 times over a week. Relative standard deviation (RSD) of the mean recovery for samples under study was 2.1 % indicating good intermediate precision.

3.2.3.4 The limits of detection (LOD) and quantification (LOQ)

The limits of detection (LOD) and quantification (LOQ) were also examined. LOD was calculated as 3.3(s/S) and LOQ as 10(s/S) where s is the standard deviation for five replicates of the measurement of placebo solution, S is slope of the weighed regression of calibration equation. The LOD and LOQ obtained were 0.77 and 2.3 μg/mL, respectively, indicating good detectability. [73].

CHAPTER 4

SEQUENTIAL ININJECTION ANALYSIS SPECTROPHOTOMETRIC METHOD FOR THE ASSAY OF DICLOFENAC SODIUM IN PHARMACEUTICAL PREPARATIONS

4.1 DICLOFENAC (DCS) LITERATURE REVIEW

Diclofenac, [o-[(2, 6-dichlorophenyl) amino] phenyl] acetic acid (Scheme 4) belongs to a class of nonsteroidal anti-inflammatory drugs (NSAIDs). In pharmacologic studies, diclofenac has shown anti-inflammatory, analgesic, and antipyretic activity. As with other NSAIDs, its mode of action is not know; its ability to inhibit prostaglandin synthesis, however, may be involved in its efficacy in relieving pain related to inflammation and primary dysmenorrheal. With regard to its analgesic effect, diclofenac is not a narcotic. Diclofenac is used in treating osteoarthritis, rheumatoid arthritis, and ankylosing spodylitis. Due to its low solubility [74] it is commercially available as its sodium salt. A number of analytical methods have been developed for the quantitative determination of this drug in dosage forms and in biological samples these methods includes:

Spectrophotometry, J. A. Rodrigues and et al. described spectrophotometry methods utilizing SIA technique this methods based on the oxidation of Diclofenac with potassium permanganate in alkaline medium and the measurement of the green product formed [75].

Snezana Mitic et al. described kinetic method for the determination of micro quantities of diclofenac sodium (DS) The method is based on a ligand-exchange reaction. The reaction was followed spectrophotometrically by monitoring the rate of appearance of the cobalt diclofenac complex at 376 nm [76].

Marcelo M. Sena et al. proposed a simple and rapid analytical procedure for determination of diclofenac (DCF) in the presence of B vitamins, based on UV measurements [77]

Mahmoud Mohamed Issa et al. developed highly sensitive indirect atomic absorption spectrophotometric (AAS) methods for the determination of Diclofenac sodium and some other pharmaceutical. The method is based on the oxidation of the drugs with iron (III), the excess of iron (III) was extracted into diethyl ether and then the iron (II) in the aqueous layer was aspirated into an air-acetylene flame and determined by AAS. [78].

Agatonović-Kustrin S et al. developed a new spectrophotometric method in which diclofenac sodium is analyzed and determined as its Fe (III) complex. The method is based on the reaction of Diclofenac sodium with Fe (III) chloride, in the presence of ammonium thiocyanate, in the pH range 4.2-6.5, forming a red chloroform extractable (2:1) complex with maximum absorbance at 481 nm. [79].

Souza, et al. proposed a modified procedure for the visible spectrophotometric determination of diclofenac, in pharmaceutical preparations using as reagent an aqueous solution of copper (II) the method is based on the monitoring of the green color complex which formed between copper(II) and diclofenac with a maximum light absorption at 680 nm [80].

Fluorometry methods were described by: Marcela A. et al. developed a spectrofluorometric method for the microdetermination of diclofenac sodium. The method is based on its reaction with cerium (IV) in an acidic solution and measurement of the fluorescence of the Ce (III) ions produced. [81].

A.M. Pimenta, et al. described method for the determination of diclofenac sodium. The method is based on the concept of sequential injection analysis [82].

Carreira LA et al. developed a new method for the determination of Diclofenac sodium in bulk and in pharmaceutical preparations. The method is based on using of Eu^{3+} ions as the Fluorescent probe. The technique was built around the hypersensitive property of the transitions of the fluorescent probe ion, Eu^{3+}, at 616 nm [83].

Arancibia JA et al. Studied the complex formed between alpha-cyclodextrin (CD) and the anti-inflammatory drug diclofenac in aqueous solution and also on its potential analytical applications. It was corroborated that the fluorescence emission band of diclofenac is significantly intensified in the presence of alpha-CD [84]

Chromatography: Brett J. et al. determined Diclofenac sodium and some other pharmaceutical in water by Isotope Dilution Liquid Chromatography/Tandem Mass Spectrometry, The method employs solid phase extraction (SPE) and liquid chromatography/tandem mass spectrometry (LC-MS/MS), using electrospray ionization (ESI) in both positive and negative modes. [85].

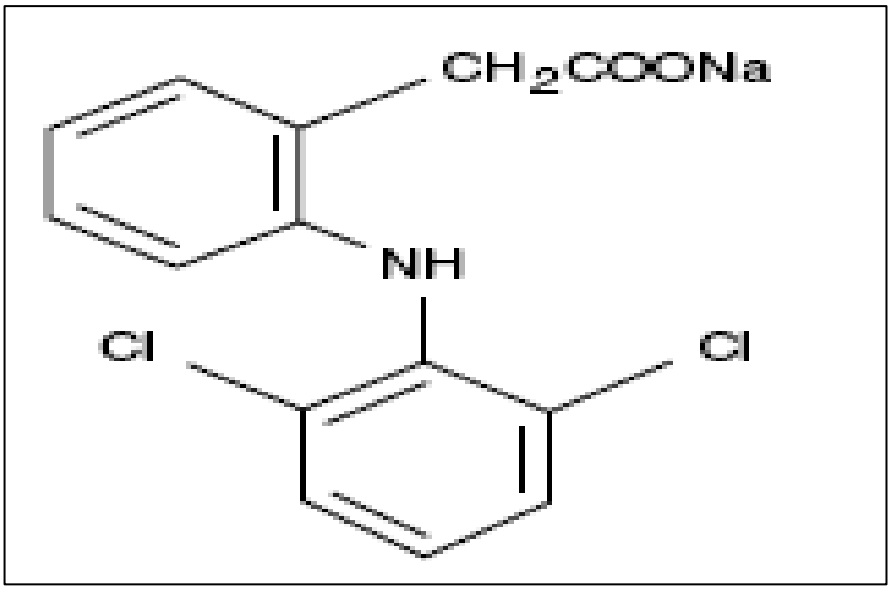
Anping Deng et al. described residue Analysis of the Pharmaceutical Diclofenac in Different Water Types Using enzyme-linked immunosorbent assay (ELISA) and GC−MS. [86]

Potentiometric: British pharmacopoeia described potentiometric method for the determination of dicolofenac sodium in tablet formulation; the method is based on dissolving of 0.250 g of the tablet powder in 30 ml of anhydrous acetic acid, titration of this solution against 0.1 M perchloric acid and determining the end-point potentiometercally [87].

Zholt Kormosh et al. described Potentiometric determination of diclofenac in pharmaceutical formulation by membrane electrode based on ion associate of diclofenac with base dye Safranine T Fig. 35 (b) an using this ion associate as an electrode active substance for membrane electrode. [88]

Electrochemical sensors: Z. Kormosh et al. prepared a novel diclofenac ion-selective electrode. [89]

Zholt Kormosh et al. developed a new diclofenac-selective electrode based on an ion associate of diclofenac with a basic dye BIK as a membrane carrier [90]



Scheme 4: Diclofenac sodium

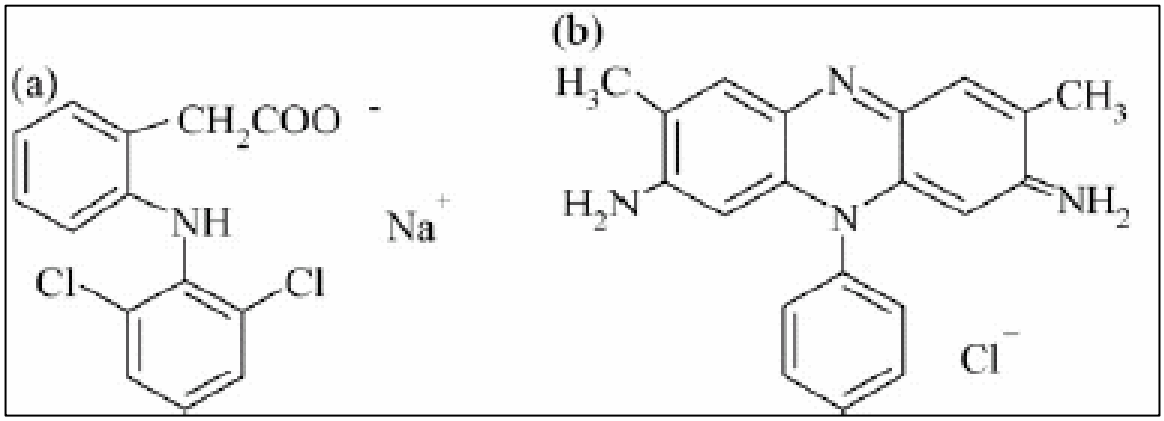


Figure 35 (a) Diclofenac sodium and (b) Safranine T

4.2 RESULTS AND DISCUSSION

4.2.1 Reaction Mechanism

The chemical system is based on the reaction of permanganate with diclofenc sodium in a slightly acidic medium of 6.0 x 10^{-6} mol l^{-1} sulfuric acid and monitoring the absorbance peaks produced at 450 nm for the oxidized form of the drug utilizing the SIA technique. The chemical system is based on the reaction of permanganate with diclofenc sodium in a slightly acidic medium of 6.0 x 10^{-6} mol l^{-1} sulfuric acid and monitoring the absorbance peaks produced at 450 nm for the oxidized form of the drug utilizing the SIA technique. Permanganate appearing as four peaks due to the different oxidation forms of what?? as in plot 2. The UV absorption spectrum of the tetrahedral d0 complex MnO_4^{2-} has become a prototype spectrum in transition-metal spectroscopy; a well-resolved experimental spectrum that has been published in 1967 by Holt and Ballhausen [91]. This prototype spectrum is attributed to the vibronic features of the absorption spectrum of permanganate that can be reproduced within this vibronic coupling scheme, including the vibrational structure due to Jahn-Teller active normal modes that lead to minima at distorted (lower symmetry) geometries [91-92]. The spectra illustrated in Fig. 36 clearly show that the reaction was found to be time dependent. The absorbance decreases while the absorbance maxima of the oxidized form of the drug increase by time as in the same figure (plots 3 to 8) thus resulting in a well defined isosbestic point (Fig. 36).

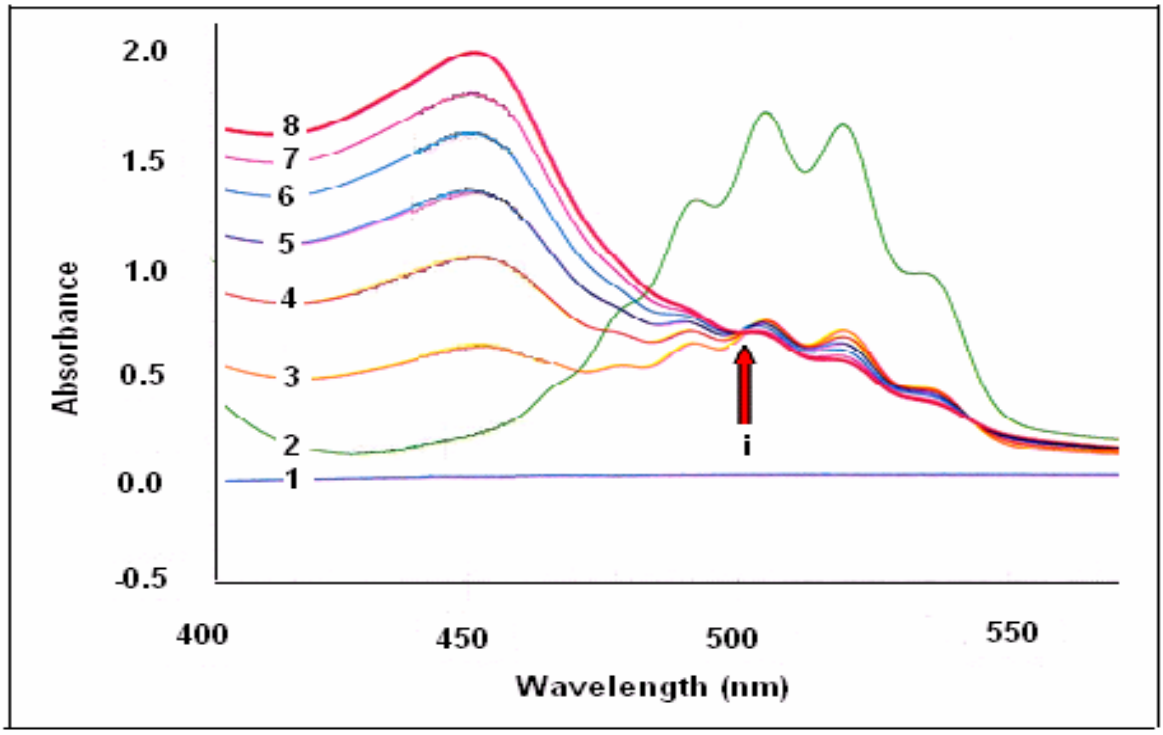


Figure 36 Absorption spectra of various solutions (1 = blank; 2= a solution of pure potassium Permanganate, 3 to 8= spectra of a mixture of DCS and KMnO4 at 0s, 40s, 80s, 120s, 160s, and 200s, respectively). i= shows the isosbestic point.

However, the absorbance of DCS-oxidized form monitored at the wavelength of 450 nm was found to be reasonable and measurable after a delay time of 30 seconds. It is worth mentioning here that SIA technique is unique in measuring the absorbance values at a fixed time with extremely high precision in comparison with the traditional spectrophotometry.

The Electron paramagnetic resonance (EPR) spectrum was scanned for the reaction products after completion (Fig. 37). The plot is showing very stable sixtet hyperfine splitting indicative of the well known EPR signal feature of manganese (II) [93]. Additionally very weak triplet of sixtet hyperfine splitting were also observed corresponding to the oxidized form of the drug system. Around g = 2.002, superimposed radical feature peak appeared, presumably due to the oxidized form of the drug as a diradical species. This diradical species was confirmed by performing another EPR scan for the drug with a milder cerium (IV) oxidant in acidic medium. This diradical species was found to too weak to be considered for any quantitative measurements.

It is therefore all quantitative measurements and investigations were carried out by monitoring the absorbance of another oxidized species of the drug at 450 nm which is believed to be the quinone imide stable compound in acidic medium [75] that is shown by the mechanism illustrated in scheme 5

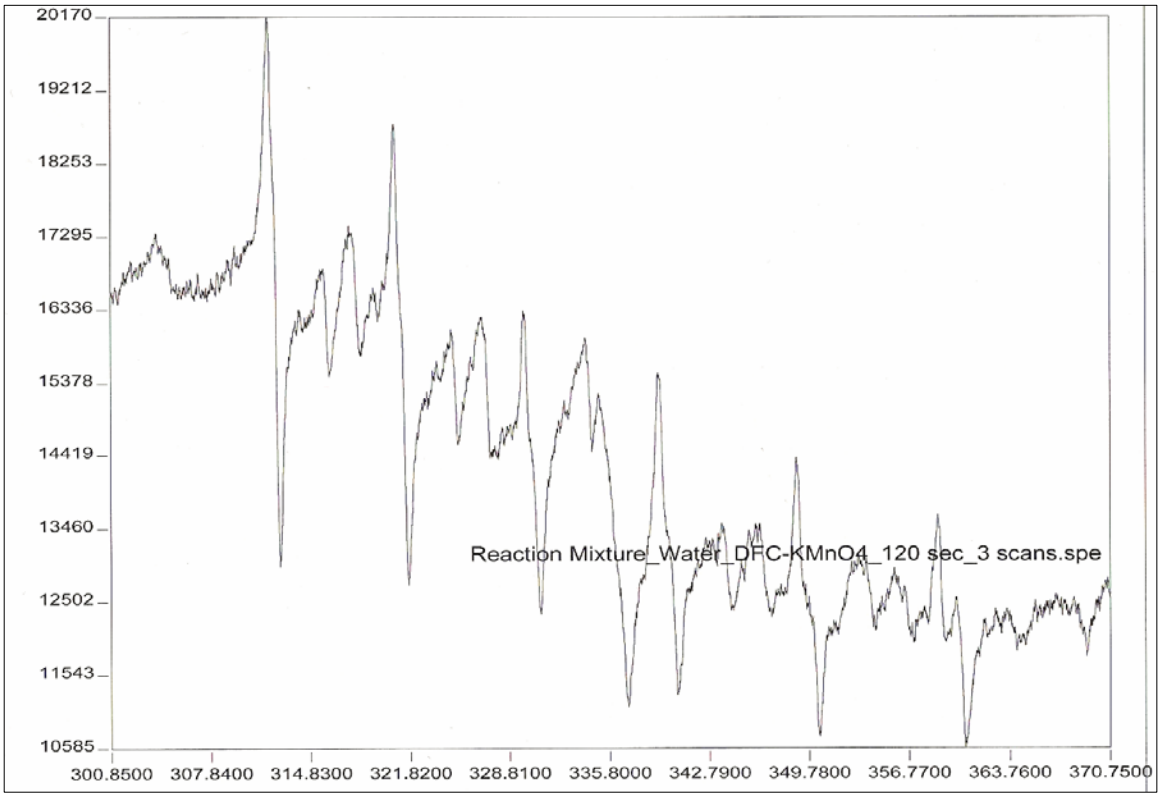
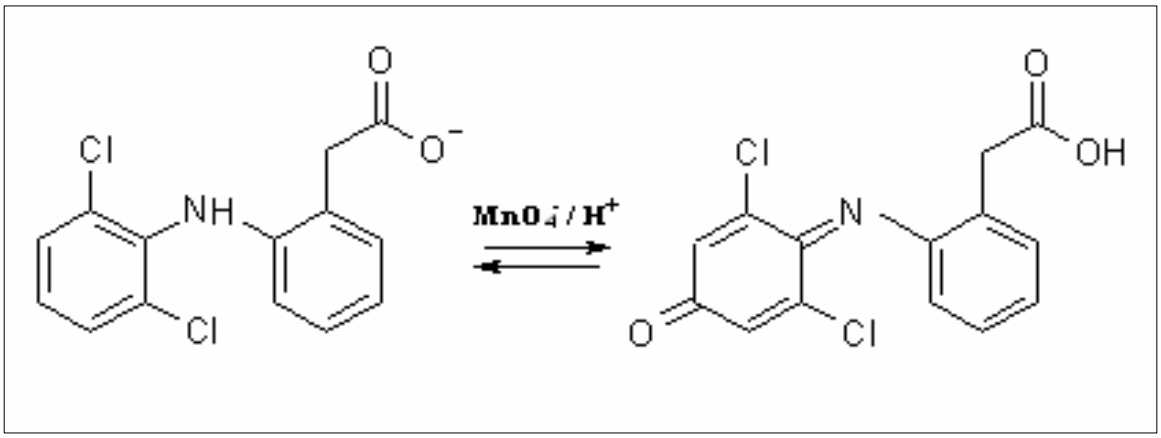


Figure 37 Electron Paramagnetic Resonance (EPR) spectrum for a mixture of Diclofenac sodium and potassium permanganate.



Scheme 5: Proposed reaction mechanism for the oxidation of Diclofenac sodium with potassium permanganate in acidic media

4.2.2 Method optimization

The results obtained by the present SIA method for the assay of DCS after oxidation with permanganate was found to be affected by the change in the permanganate concentration and the flow rate of aspiration and dispensing. The simple univariate type of optimization was utilized for this purpose as manifested below.

4.2.2.1 Optimization of the permanganate concentration

The univariant, one-variable-at-a-time, in this concern, a number of experiments were conducted using various potassium permanganate concentrations (50, 100, 150, 200, 250, 350, 450, and 500 mg l^{-1}) in acidic medium, keeping the acid concentration and the flow rate constants at 6 x 10^{-6} mol l^{-1} and 5 µl s^{-1}, respectively. The absorbance of the brown color produced at 450 nm was found to increase by increasing potassium permanganate concentration (Fig.38 and Table 16). The optimum potassium permanganate concentration was considered to be 250 mgl^{-1} as the increase of the absorbance was not highly significant beyond this concentration. However, though the absorbance kept increasing with potassium permanganate concentration more than 250mgl^{-1}, there is fear that the tubing of the SIA system would be affected.

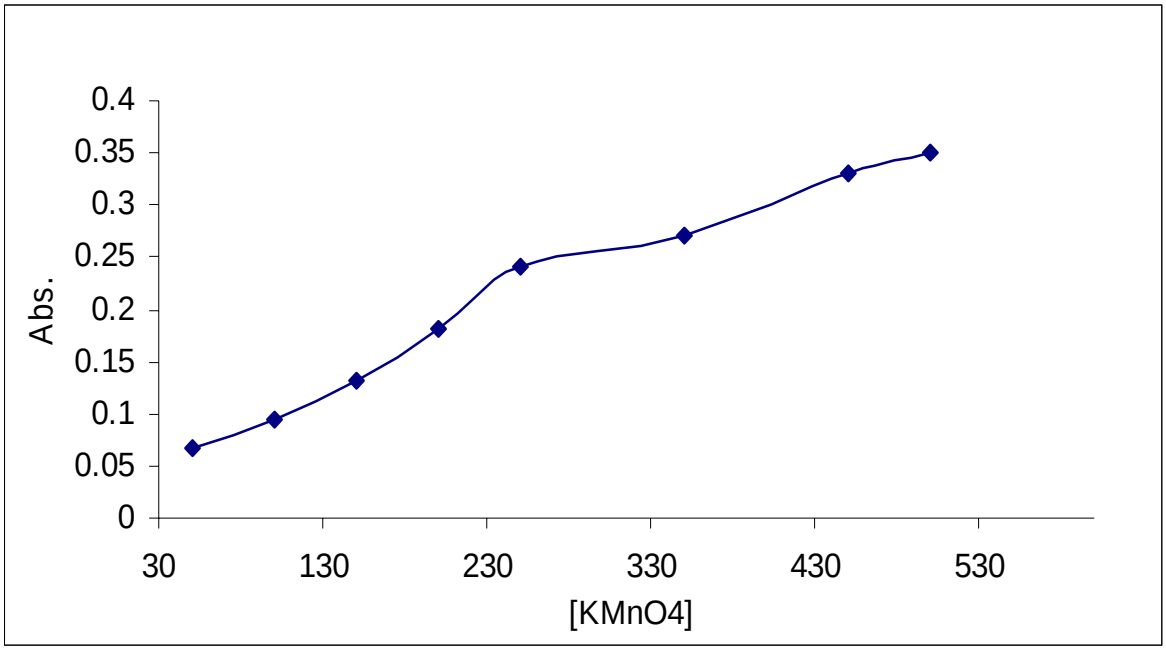


Figure 38 The effect of $KMnO_4$ concentration on the absorbance (430 nm) of a solution of 30 mgl^{-1} DCS. Absorbance recorded was an average of three determinations

Table 16: The Effect of $KMnO_4$ concentration on the absorbance (430 nm) of DCS

Exp. No.	$KMnO_4$ (ppm)	Absorbance*
1	50	0.067
2	100	0.095
3	150	0.132
4	200	0.181
5	250	0.24
6	350	0.27
7	450	0.33
8	500	0.35

*Average of three determinations

4.2.2.2 Optimization of the Flow Rate

Again, the univariant type of optimization was performed for determining the suitable flow rate for quantitative assay of the DCS. Series of experiments were conducted using different flow rates ranging between 5 and 25 μls^{-1}, keeping the acid and permanganate concentrations constants at 6 x 10^{-6} M and 250mgl^{-1}, respectively. The absorbance of the brown color produced at 450 nm was found to decrease by increasing the flow rate (Table 17 and Fig. 39). The decrease in the absorbance became insignificant when the flow rate exceeded 15 μls^{-1}. This observation is in quite agreement with the fact that the reaction is time dependent. The optimum flow rate for the proposed SIA method was considered to be 5 μls^{-1}.

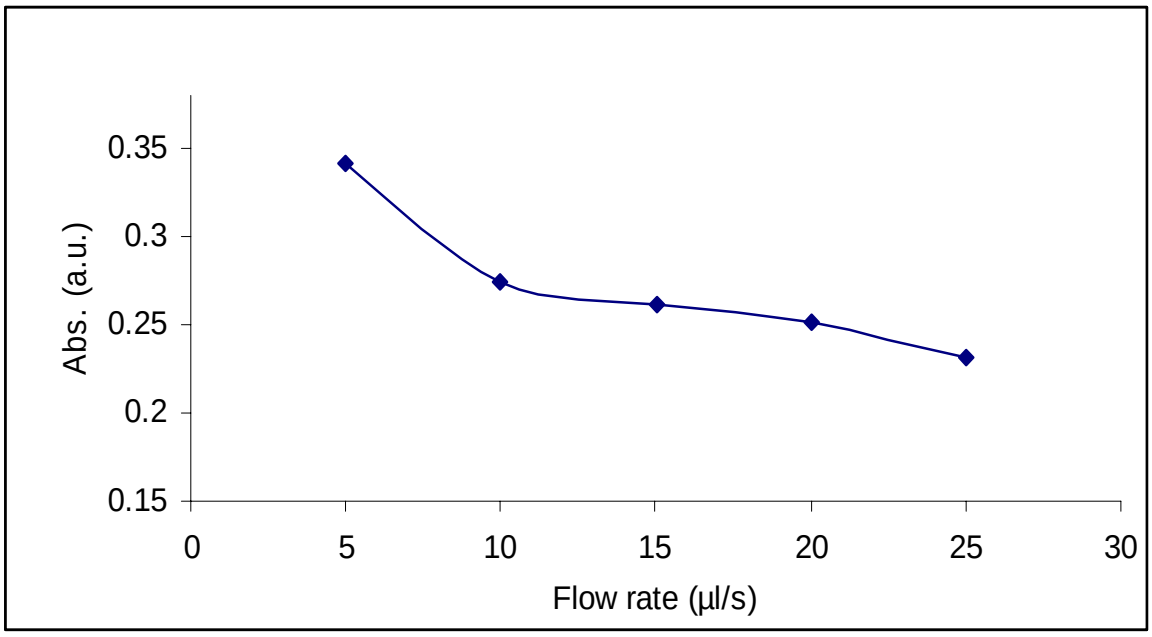


Figure 39 The effect of the flow rate on the absorbance (430 nm) of 30 mgl^{-1} DCS. Absorbance recorded was an average of three determinations.

Table 17: The Effect of the flow rate on the absorbance of DCS

Exp. No.	Flow rate (µl/s)	Absorbance*
1	5	0.341
2	10	0.275
3	15	0.262
4	20	0.252
5	25	0.232

*Average of three determinations

4.2.3 Analytical Appraisal

Validation of the compendial procedure must meet proper standards of accuracy and reliability supported by sufficient data to document their validity. Diclofenac sodium raw material and in dosage form is determined by potentiometry in the BP 2007 [87]. The method is internally validated following the USP30-NF25 procedure. The following typical analytical performance characteristics are considered: linearity and range, limit of detection, limit of quantitation, accuracy, precision, specificity, and robustness.

4.2.3.1 Linearity and Range

The linearity of calibration curves obtained from the optimized SIA method set here has been tested over a range of 15 – 150 mg l^{-1} standard solutions of diclofenac sodium in the absence of the drug matrix (excepients) components. Beer's law was found to be obeyed over a concentration range of 30–135 μg ml^{-1} with a regression equation of y = 0.0987 + 0.00426 x and correlation coefficient of better than 0.998 Table 18 and Fig.40 show a typical SIA calibration obtained under the optimum conditions by triplicate consequent injection of the DCS standard solutions.

Table 18 Diclofenac sodium calibration obtained from the optimized SIA method

Concentration of Diclofenac	**Absorbance**	**Acceptance criteria**
mg/ml		
15	0.180	
30	0.226	
45	0.282	
60	0.354	
75	0.427	
90	0.491	
105	0.540	
120	0.615	
135	0.666	
150	0.700	
Regression: $y = ax + b$ $a = 0.00426$ $b = 0.0987$ $r^2 = 0.998$		0.998 – 1.002

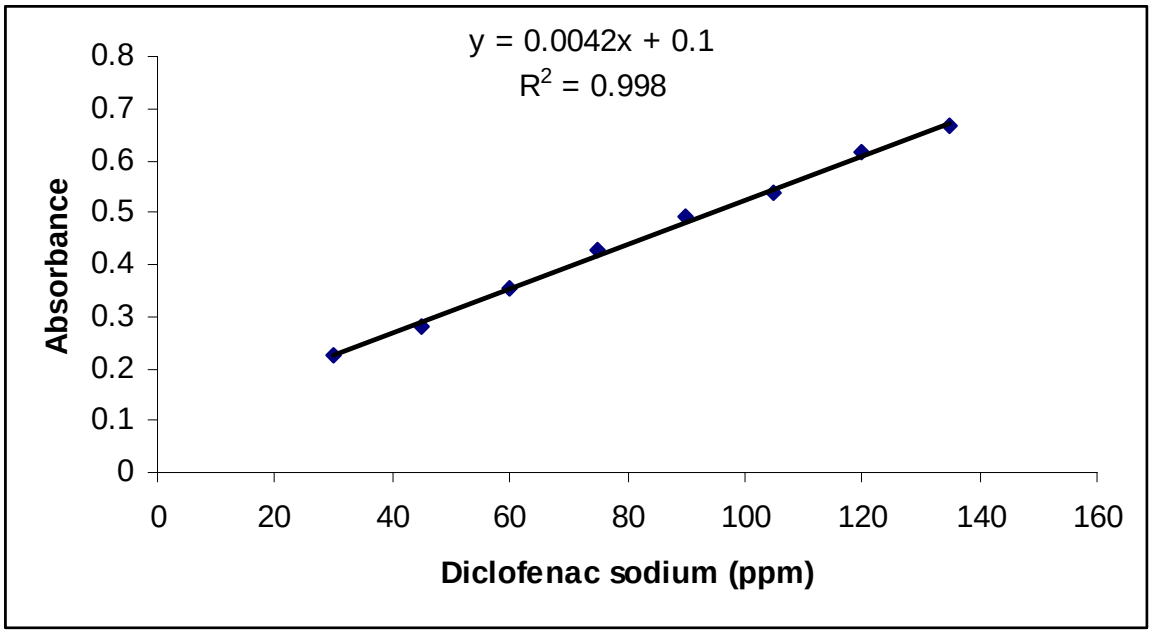


Figure 40: The Absorbance of the DCS at 450 nm versus concentration.

4.2.3.2 *The limits of detection (LOD) and limit of quantification (LOQ)*

The limits of detection (LOD) and limit of quantification (LOQ) were also examined. LOD was calculated as 3.3(s/S) and LOQ as 10(s/S) where s is the standard deviation for nine replicates of the measurement of the blank solution, S is slope of the weighed regression of calibration equation. The LOD and LOQ obtained were 0.24 and 0.7 μgml^{-1}, respectively, indicating good detectability [73].

4.2.3.3 Accuracy

The accuracy of an analytical procedure is usually established across the linearity range of the calibration curves. The validation is conducted chemometrically by comparing the results obtained from the new SIA method with those obtained from the standard BP 2007 [87] method. In this respect, the same batches of samples containing diclofenac sodium in dosage forms were quantitatively analyzed for the active drug by both, the new SIA method and by the BP standard method. The percentage recovery, standard deviation and finally the t-test values were calculated. Thus, a minimum of 9 determinations over a minimum of 3 concentration levels covering a specified range (e.g. 3 concentrations/3 replicates each of the total analytical procedure).

The proposed SIA method was used to determine Diclofenc sodium in proprietary pharmaceutical formulations (tablet). Table 19 shows the results obtained. The SIA results obtained were statistically compared by the results obtained for the assay of the same

samples of Diclofenc sodium in tablet formulations following the British Pharmacopoeia standard method [87].

For each sample type, the mean concentrations (n = 5) of Diclofenc sodium obtained with the two methods were compared using the student t-test to evaluate for the accuracy of the new SIA method as in Tables 19 and 20. The t-test values were always less than the tabulated values indicating high accuracy of the new method. In addition to that the results obtained proved no interference occurred from excipients already found in their pharmaceutical preparations.

Table 19 Diclofenac sodium accuracy results for the proposed SIA methods

Conc. of Diclofenac µg/ml	**Absorbance**	**Recovery (%)**	**Acceptance Criteria**
30	0.235	100.77	
30	0.216	98.24	
30	0.226	96.72	
60	0.360	102.33	
60	0.353	97.47	
60	0.349	98.89	
90	0.497	102.35	
90	0.490	100.55	
90	0.485	99.49	
Mean (recovery)		: 99.6	98.0–102.0 %
Standard deviation		: 2.01	
Relative standard deviation (RSD)		: 2.02 %	$\leq$ 2 %

Table 20 Results obtained by the SIA and British Pharmacopoeia methods for the analysis of Diclofenac sodium in tablets samples

Drug	Supplier	Contents (mg)	Mean recovery ± RSD (%)[a]		t[b]
			SIA method	BP[c] method	
Olfen-50	Mepha Ltd., Aesch-Basel, Switzerland	Diclofenac sodium (50)	97.8 ± 1.3	99 ± 0.15	2.1
Olfen-25	Mepha Ltd., Aesch-Basel, Switzerland	Diclofenac sodium (25)	98.6 ± 1.5	99 ± 0.16	2.2
INFLA-BAN 50	The Arab Pharmaceutical Manufacturing Co. Ltd., Sult-Jordan	Diclofenac sodium (50)	98.3 ± 1.8	99.8 ± 0.14	1.6
INFLA-BAN 25	The Arab Pharmaceutical Manufacturing Co. Ltd., Sult-Jordan	Diclofenac sodium (25)	98.8 ± 1.8	99.9 ± 0.14	1.8
Retard-100	Dar Al Dawa, Na'ur - Jordan	Diclofenac sodium (100)	99.2 ± 2.0	99.7 ± 0.15	2.0
Divido	Tabuk Pharmaceutical Mfg. Co., Tabuk, Saudi Arabia	Diclofenac sodium (75)	98.5 ± 1.6	99.6 ± 0.17	2.1

[a] Relative standard deviation for 5 replicates

[b] Student t-test values (t Critical = 2.9)

[c] British Pharmacopoeia

4.2.3.4 Precision

The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation of a series of measurements. Precision may be a measure of either the degree of repeatability (short-term precision) or reproducibility (intermediate or long term precision) of the analytical procedure under normal operating conditions.

4.2.3.4.1 Repeatability

The short term precision for DCS was demonstrated as the relative standard deviation, RSD, of nine samples (3 replicates each) of 60.0 μg ml^{-1} DCS (Table 21). The RSD found was 1.1% , a value that lies within the acceptance criteria (<2%).

Another set of nine samples of DCS was analyzed again according to the optimum procedure but at another day (Table 22). The results indicate once again that the repeatability of the method is acceptable.

Table 21 Diclofenac sodium repeatability results for the proposed SIA methods

Sample no.	Concentration (μg/ml)	Absorbance
1	60.0	0.364
2	60.0	0.368
3	60.0	0.356
4	60.0	0.366
5	60.0	0.364
6	60.0	0.357
7	60.0	0.363
8	60.0	0.630
9	60.0	0.361
Mean : 0.362		
Standard deviation : 0.004		
Relative standard deviation (RSD) : 1.1 %		

Table 22 Diclofenac sodium repeatability results for the proposed SIA methods

Sample no.	**Quantity added (mg ml^{-1})**	**Absorbance**
1	60.0	0.350
2	60.0	0.355
3	60.0	0.347
4	60.0	0.346
5	60.0	0.345
6	60.0	0.337
7	60.0	0.353
8	60.0	0.352
9	60.0	0.345
Mean	:	0.348
Standard deviation	:	0.005
Relative standard deviation (RSD)	:	1.5 %

4.2.3.4.2 Reproducibility

The intermediate precision for the optimized method was demonstrated by analyzing nine DCS samples (3 replicates each) over different days. The results obtained (Table 23) indicate a RSD of 1.8% that, also, lies within the acceptable range of precision.

Table 23 Diclofenac sodium precision results for the proposed SIA methods

Sample no.	Concentration (mg/ml)	Absorbance
1	60.0	0.365
2	60.0	0.345
3	60.0	0.347
4	60.0	0.351
5	60.0	0.349
6	60.0	0.350
7	60.0	0.348
8	60.0	0.351
9	60.0	0.343
Mean		: 0.350
Standard deviation		: 0.006
Relative standard deviation (RSD)		: 1.8 %

CONCLUSION

The SIA is considered as the technique, prime goal to be achieved and demonstrated during this work for quantitative assays of two important drugs, that is Ketoconazole and Diclofenac sodium.

The experimental results obtained spurred into the development of two new methods for the quantitative assay of the antifungal drug ketoconazole and anti-inflammatory Diclofenc Sodium. The methods are suitable for the assay of the drugs in generic forms pharmaceutical preparations such as tablet and suspension formulations.

The newly adopted methods have the advantage over the official British Pharmacopoeia and other reported standard methods with respect to rapidity, economy automation using the latest-state-of-the-art Sequential Injection Analysis (SIA) technology.

Also this new system was successfully used for the optimization of instrumental and chemical different parameters such as delay time in the holding coil, flow rate of the colored products through the flow cell, concentration of sulfuric acid, concentration of potassium permanganate, and concentration of cerium (IV). The optimization was achieved utilizing different chemometrics approaches namely, uni-variate, factorial design, and MultiSimplex.

The optimized SIA methods were applied to the assay of Ketoconazole and Diclofenac sodium in tablet forms collected from local dispensaries. The results obtained showed good accuracy and precision.

The application of the SIA technique would be of a great advantage to the industrial sectors in the kingdom especially those in the pharmaceutical arena.

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APPENDICES

(I) OPTIMIZED COMPUTER FLOW PROGRAM USED FOR SIA SYSTEM CALIBRATION

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 2

syringe pump Aspirate (microliter) 100

syringe pump Delay Until Done

Syringe Pump Flowrate (microliter/sec) 20

Valve port 1

chemical New Sample

chemical Name std1

chemical Quantity 50

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 3

syringe pump Aspirate (microliter) 100

syringe pump Delay Until Done

Syringe Pump Flowrate (microliter/sec) 20

Valve port 1

chemical New Sample

chemical Name std2

chemical Quantity 100

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 4

syringe pump Aspirate (microliter) 100

syringe pump Delay Until Done

Syringe Pump Flowrate (microliter/sec) 20

Valve port 1

chemical New Sample

chemical Name std3

chemical Quantity 150

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 5

syringe pump Aspirate (microliter) 100

syringe pump Delay Until Done

Syringe Pump Flowrate (microliter/sec) 20

Valve port 1

chemical New Sample

chemical Name std4

chemical Quantity 200

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 6

syringe pump Aspirate (microliter) 100

syringe pump Delay Until Done

Syringe Pump Flowrate (microliter/sec) 20

Valve port 1

chemical New Sample

chemical Name std5

chemical Quantity 250

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 7

syringe pump Aspirate (microliter) 100

syringe pump Delay Until Done

Syringe Pump Flowrate (microliter/sec) 20

Valve port 1

chemical New Sample

chemical Name unknown 1

chemical Quantity unknown

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

(II) OPTIMIZED COMPUTER FLOW PROGRAM FOR KETOCONAZOLE DETERMINATION

‘STANDARD 1

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 2

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 3

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

Syringe Pump Flowrate (microliter/sec) 16.202

Valve port 1

chemical New Sample

chemical Name std1

chemical Quantity 60

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

‘STANDARD 2

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 2

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 4

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

Syringe Pump Flowrate (microliter/sec) 16.202

Valve port 1

chemical New Sample

chemical Name std2

chemical Quantity 90

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

‘STANDARD 3

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 2

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 5

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

Syringe Pump Flowrate (microliter/sec) 16.202

Valve port 1

chemical New Sample

chemical Name std3

chemical Quantity 120

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

‘STANDARD 4

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 2

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 6

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

Syringe Pump Flowrate (microliter/sec) 16.202

Valve port 1

chemical New Sample

chemical Name std4

chemical Quantity 150

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

‘STANDARD 5

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 2

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 7

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

Syringe Pump Flowrate (microliter/sec) 16.202

Valve port 1

chemical New Sample

chemical Name std5

chemical Quantity 180

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

‘UNKNOWN

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 2

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

syringe pump Flowrate (microliter/sec) 100

Valve port 8

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

Syringe Pump Flowrate (microliter/sec) 16.202

Valve port 1

chemical New Sample

chemical Name std6

chemical Quantity unknown

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

(III) OPTIMIZED COMPUTER FLOW PROGRAM USED FOR DICLOFENAC SODIUM DETERMINATION

"Run 1 -50 ppm

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

'Permanganate

syringe pump Flowrate (microliter/sec) 100

Valve port 2

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

'Diclofenac

syringe pump Flowrate (microliter/sec) 100

Valve port 3

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

Valve port 1

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Delay (sec) 30

Syringe Pump Flowrate (microliter/sec) 10

Valve port 1

chemical New Sample

chemical Name std1

chemical Quantity 15

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

"Run 2 -30 ppm

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

'Permanganate

syringe pump Flowrate (microliter/sec) 100

Valve port 2

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

'Diclofenac

syringe pump Flowrate (microliter/sec) 100

Valve port 4

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

Valve port 1

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Delay (sec) 30

Syringe Pump Flowrate (microliter/sec) 10

Valve port 1

chemical New Sample

chemical Name std2

chemical Quantity 30

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

"Run 3 -45 ppm

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

'Permanganate

syringe pump Flowrate (microliter/sec) 100

Valve port 2

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

'Diclofenac

syringe pump Flowrate (microliter/sec) 100

Valve port 5

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

Valve port 1

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Delay (sec) 30

Syringe Pump Flowrate (microliter/sec) 10

Valve port 1

chemical New Sample

chemical Name std3

chemical Quantity 45

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

"Run 4 -60 ppm

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

'Permanganate

syringe pump Flowrate (microliter/sec) 100

Valve port 2

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

'Diclofenac

syringe pump Flowrate (microliter/sec) 100

Valve port 6

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

Valve port 1

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Delay (sec) 30

Syringe Pump Flowrate (microliter/sec) 10

Valve port 1

chemical New Sample

chemical Name std4

chemical Quantity 60

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

"Run 5 -75 ppm

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

'Permanganate

syringe pump Flowrate (microliter/sec) 100

Valve port 2

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

'Diclofenac

syringe pump Flowrate (microliter/sec) 100

Valve port 7

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

Valve port 1

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Delay (sec) 30

Syringe Pump Flowrate (microliter/sec) 10

Valve port 1

chemical New Sample

chemical Name std5

chemical Quantity 75

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

"Run 6 -90 ppm

syringe pump Flowrate (microliter/sec) 100

Syringe Pump Valve In

Syringe Pump Fill

Syringe Pump Delay Until Done

Valve port 1

Syringe Pump Valve Out

Syringe Pump Dispense (microliter) 2000

Syringe Pump Delay Until Done

'Permanganate

syringe pump Flowrate (microliter/sec) 100

Valve port 2

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

'Diclofenac

syringe pump Flowrate (microliter/sec) 100

Valve port 8

syringe pump Aspirate (microliter) 50

syringe pump Delay Until Done

Valve port 1

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Syringe Pump Aspirate (microliter) 5

Syringe Pump Dispense (microliter) 5

Delay (sec) 30

Syringe Pump Flowrate (microliter/sec) 10

Valve port 1

chemical New Sample

chemical Name std6

chemical Quantity 90

syringe pump Dispense (microliter) 1500

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

Vita

Hatim D. Mohamed graduated from the Chemistry Department, University of Khartoum Sudan with a second class 2001. He worked as a lab chemist in Central Petroleum Laboratories (CPL), Khartoum, Sudan. He joined the King Fahd University of Petroleum & Minerals 2006 as a Research Assistant.